Filipa Maria Fortio Mourato Teixeira Grosso Ledo

MULTI-LEVEL ANALYSIS OF CARBAPENEM-RESISTANT *ACINETOBACTER* SPP.: TOWARDS AN UNDERSTANDING ON POPULATION DYNAMICS

Tese do 3º Ciclo de Estudos Conducente ao Grau de Doutoramento em Ciências Farmacêuticas na Especialidade de Microbiologia

Tutors:

Professora Doutora Luísa Peixe

REQUIMTE
Departamento de Microbiologia,
Faculdade de Farmácia
Universidade do Porto, Portugal

Professora Doutora Sandra Quinteira

Centro de Investigação em Tecnologias da Saúde, Vila Nova de Famalicão.
Centro de Investigação em Biodiversidade e Recursos Genéticos, Vairão.
Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, Portugal.

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Multi-level analysis of carbapenem-resistant *Acinetobacter* spp.: towards an understanding on population dynamics

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Abstract

Organisms belonging to *Acinetobacter* genus constitute a remarkable example of bacterial evolution, evolving from traditionally harmless organisms towards to important nosocomial pathogens, probably in consequence of the introduction of new powerful antibiotics. In the last decades we have assisted to increasing reports of drug-resistant hospital related, and more recently, community acquired infections, with *Acinetobacter baumannii* being the most prevalent species of this genus in the hospital setting. In addition, the prevalence of non-*baumannii Acinetobacter* species involved in nosocomial infections has increased in the last years, probably due to the exchange of resistance determinants among *Acinetobacter* species. Carbapenem resistance is of particular concern, since this class of antibiotics constitutes the first line therapy for *Acinetobacter* infections; however, its use has become limited by the increasing prevalence of resistant strains. The recognition of the main carbapenem-resistant *A. baumannii* lineages responsible for nosocomial infections as well as the complete understanding of the factors that are driving their dissemination and persistence is essential to create strategies to control these organisms.

This study focused on the characterization of the population structure of carbapenem-resistant *A. baumannii* (CRAB) clinical isolates from Portugal, Brazil and Czech Republic. The mechanisms underlying carbapenem resistance, not only in *A. baumannii* but also in other *Acinetobacter* species (*A. haemolyticus* and *A. lwoffii*), the role of mobile genetic elements in the inter- and intra-species dissemination of carbapenem resistance, and the comparison of adhesion and biofilm abilities among the main Portuguese CRAB lineages were explored.

All the typing methods used to characterize *A. baumannii* clinical isolates revealed that very few lineages are associated with carbapenem-resistance, belonging most of them to European clone II. Using Bartual MLST scheme, which provided a higher discrimination, was possible to identify three main lineages in Portugal: ST92 carrying *bla*$_{\text{OXA-23,}}$ ST98 carrying *bla*$_{\text{OXA-24/40}}$, both belonging to the same clonal complex (CC) 92, and ST103 carrying *bla*$_{\text{OXA-58}}$, a lineage with no apparent relationship with CC92. The use of this MLST scheme also allowed the detection of a progressive switching, with ST98 carrying *bla*$_{\text{OXA-24/40}}$ being gradually replaced by ST92 carrying *bla*$_{\text{OXA-23}}$, a lineage that also included a pandrug resistant isolate. Carbapenem resistance in Czech Republic's isolates seems to be associated with impaired CarO porin expression, but they also belonged to ST92, confirming the worldwide dissemination of this lineage. On the other hand, Brazilian *bla*$_{\text{OXA-23}}$-carrying *A. baumannii* clinical isolates were distributed among four new STs: ST131, ST132, ST133 and ST134, suggesting a local

diversity hotspot. This finding, together with the chromosomal location of *bla*$_{OXA-23}$ embedded in Tn*2006* observed in all *bla*$_{OXA-23}$-carrying isolates, both from Portugal and Brazil, raises the question about when or where the diversification from a common ancestral and *bla*$_{OXA-23}$ acquisition might have occurred. The CHDL genes *bla*$_{OXA-24/40}$ and *bla*$_{OXA-58}$ were found both in the chromosome and plasmids. The *bla*$_{OXA-58}$ gene was flanked by IS*Aba3*-like and IS*Aba3* elements upstream and downstream, respectively. Plasmidic *bla*$_{OXA-24/40}$ gene was found to be flanked by XerC/XerD-like binding sites which might be responsible for their mobilization by a mechanism of recombination. Only two types of *bla*$_{OXA-24/40}$ carrying plasmids were detected: 30-kb *repA_AB* (belonging to homology group GR12) identified both in *A. baumannii* and *A. haemolyticus*, and 10-kb *repAci2* (belonging to homology group GR2) identified only in *A. baumannii*. These findings are in accordance to what was observed in other countries, although associated with isolates belonging to different STs, stressing the plasmid contribution in the dissemination of carbapenem resistance and highlight interspecies plasmid transfer. Moreover, analysis of different plasmid scaffolds suggests frequent recombinatorial events on these plasmids.

The detection of an environmental *A. lwoffii* isolate harboring the metallo-β-lactamase IMP-5 raises concerns about the potential of environmental non-*baumannii* *Acinetobacter* species to acquire resistance determinants and/or act as reservoirs of resistance genes, particularly because *bla*$_{IMP-5}$ was embedded in a Tn*402*-like transposon, a structure previously identified in *A. baumannii*.

Strong adherence properties and ability to form biofilms in abiotic surfaces were observed in most isolates from the different lineages recovered in Portuguese hospitals, a trait that together with antibiotic resistance might explain their persistence.

In summary, this work illustrates the dynamics of epidemic clones and the role of carbapenem resistance determinants in the current dominance of particular *A. baumannii* lineages, both in Portugal and at a global level. It is of note the stability of specific acquired CHDL within each lineage, although the plasmid contribution on the dissemination of *bla*$_{OXA-24/40}$ was also demonstrated. The occurrence of an environmental *bla*$_{IMP-5}$-carrying *A. lwoffii* and *bla*$_{OXA-24/40}$-carrying *A. haemolyticus* stresses the possible contribution of non-*baumannii* *Acinetobacter* species in the dissemination of resistance genes. This study highlights the importance of monitoring the national and international spread of epidemic, multidrug-resistant, and virulent clones, aiming the development of more effective guidelines in the treatment and infection control procedures.

Keywords: *Acinetobacter*, carbapenem, resistance, MLST, plasmid.

Resumo

Os microrganismos pertencentes ao género *Acinetobacter* constituem um exemplo notável de evolução bacteriana, tendo passado de tradicionalmente inofensivos para importantes patogénicos nosocomiais, provavelmente em consequência da introdução de novos antibióticos. Nas últimas décadas assistiu-se a crescentes relatos de estirpes hospitalares multi-resistentes e, mais recentemente, de infeções adquiridas na comunidade, sendo a espécie *Acinetobacter baumannii* a mais frequente em ambiente hospitalar. Além disso, a prevalência de espécies de *Acinetobacter* não-*baumannii* envolvidas em infeções nosocomiais tem aumentado nos últimos anos, provavelmente devido à transferência inter-espécies de determinantes de resistência. A resistência aos carbapenemos é particularmente preocupante, uma vez que esta classe de antibióticos constitui a primeira linha terapêutica para infeções por *Acinetobacter*, estando o seu uso limitado pela crescente prevalência de estirpes resistentes. O reconhecimento das principais linhagens de *A. baumannii* resistentes aos carbapenemos e responsáveis por infeções hospitalares, bem como a compreensão dos fatores que influenciam a sua difusão e persistência, são essenciais para o desenvolvimento de estratégias para o controlo destes microrganismos.

Este estudo incidiu sobre a caracterização da estrutura populacional de isolados clínicos de *A. baumannii* resistentes aos carbapenemos (CRAB) de Portugal, Brasil e República Checa. Foram estudados os mecanismos subjacentes à resistência aos carbapenemos, não só em *A. baumannii*, mas também noutras espécies de *Acinetobacter* (*A. haemolyticus* e *A. lwoffii*), o papel de elementos genéticos móveis na disseminação inter e intra-espécies de resistência aos carbapenemos, e a comparação das capacidades de adesão e formação de biofilme entre as principais linhagens CRAB portuguesas. Todos os métodos de tipagem utilizados para caracterizar os isolados clínicos de *A. baumannii* revelaram que muito poucas linhagens estão associadas à resistência aos carbapenemos, sendo a maioria pertencente ao Clone Europeu II. Usando o esquema de MLST desenvolvido por Bartual, o que proporcionou uma maior discriminação, foi possível identificar três linhagens principais em Portugal: ST92 produtora de OXA-23, ST98 produtora de OXA-24/40, ambas pertencentes ao mesmo complexo clonal (CC)92, e ST103 produtora de OXA-58, uma linhagem sem relação aparente com CC92. A utilização deste esquema de MLST também permitiu a deteção de uma substituição progressiva da ST98 pela ST92, uma linhagem que também inclui um isolado resistente a todos os antibióticos correntemente disponíveis. Embora a linhagem ST92 se encontre também disseminada na República Checa, confirmando a disseminação mundial desta linhagem, a resistência aos carbapenemos parece estar associada a alterações na expressão da porina CarO. Por outro lado, os isolados clínicos brasileiros de *A. baumannii* e produtores de OXA-23 distribuíram-se

por quatro novos STs (ST131, ST132, ST133 e ST134), sugerindo diversidade local. Esta constatação, juntamente com a localização cromossómica do gene *bla*$_{OXA-23}$ incorporado no Tn*2006* em todos os isolados portadores de *bla*$_{OXA-23}$ (Portugal e Brasil), levanta a questão sobre quando e onde a diversificação a partir de um ancestral comum e a aquisição de *bla*$_{OXA-23}$ terão ocorrido. Os genes *bla*$_{OXA-24/40}$ e *bla*$_{OXA-58}$ foram encontrados tanto no cromossoma como em plasmídeos. O gene *bla*$_{OXA-58}$ encontrava-se flanqueado por elementos IS*Aba3*-like e IS*Aba3* a montante e a jusante, respetivamente. O gene plasmídico *bla*$_{OXA-24/40}$ foi encontrado flanqueado por sítios de ligação XerC/XerD que poderão ser responsáveis pela sua mobilização por um mecanismo de recombinação. Foram apenas detetados dois tipos de plasmídeos a codificar a OXA-24/40: *repA_AB* com 30-kb (pertencente ao grupo de homologia GR12) identificado em *A. baumannii* e *A. haemolyticus* e *repAci2* com 10-kb (pertencente ao grupo de homologia GR2) identificado apenas em *A. baumannii*. Estes resultados estão de acordo com o que foi observado noutros países, embora associados a isolados pertencentes a diferentes STs, salientando o contributo de determinados plasmídeos na disseminação da resistência aos carbapenemos, e envolvendo diferentes espécies. Além disso, a análise de diferentes estruturas plasmídicas sugere frequentes eventos de recombinação. A detecção de um isolado de *A. lwoffii* ambiental produtor de IMP-5 levanta preocupações sobre o potencial de espécies de *Acinetobacter* não-*baumannii* ambientais em adquirir resistência e/ou atuar como reservatórios de genes de resistência, principalmente porque *bla*$_{IMP-5}$ encontra-se num transposão Tn*402*-like, previamente identificado em *A. baumannii*. Salienta-se ainda que a maioria dos isolados portugueses apresentou forte aderência e capacidade de formar biofilmes em superfícies abióticas, características que, juntamente com a resistência aos antibióticos podem explicar a sua persistência.

Em resumo, este trabalho ilustra a dinâmica de clones epidémicos e o papel dos determinantes resistência aos carbapenemos na prevalência de determinadas linhagens de *A. baumannii*, tanto em Portugal como a nível global. É digno de nota a estabilidade de determinadas carbapenemases em linhagens específicas, apesar da capacidade de disseminação de *bla*$_{OXA-24/40}$ ter sido também demonstrada. A ocorrência de um *A. lwoffii* ambiental e produtor de IMP-5 e de *A. haemolyticus* produtores de OXA-24/40 salienta a possível contribuição de espécies *Acinetobacter* não-*baumannii* na disseminação de genes de resistência. Este estudo ilustra a importância de estudar a diferentes níveis os fatores intervenientes na emergência da resistência, essencial para monitorizar a disseminação nacional e internacional de clones/plasmídeos epidémicos e de relevância para o desenvolvimento de orientações mais eficazes para o tratamento e o controlo de infeção.

Palavras-chave: Acinetobacter, carbapenem, resistência, MLST, plasmídeo.

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AFLP	Amplified fragment length polymorphism
ARDRA	Amplified rDNA restriction analysis
ATCC	American Type Culture Collection
bp	Base pairs
BSA	Bovine Serum Albumin
CC	Clonal Complex
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
CFU	Colony-Forming Units
CHDL	Carbapenem-hydrolyzing class D β-lactamases
CLSI	Clinical Laboratory Standards Institute
CRAB	Carbapenem resistant *Acinetobacter baumannii*
DEPC	diethyl pyrocarbonate
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
ESBL	Extended-spectrum ß-Lactamases
EU	European clone
EUCAST	European Committee on Antimicrobial Susceptibility Testing
h	Hours
HGT	Horizontal Gene Transfer
IS	Insertion Sequence
kV	kilovolts
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MBL	Metallo-β-lactamase
MH	Mueller-Hinton
MHT	Modified Hodge Test
MIC	Minimum Inhibitory Concentration

min	Minutes
MLST	MultiLocus Sequence Typing
OD	Optical Density
ORF	Open Reading Frame
PBP	Protein Binding Penicillin
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
pI	Isoelectric Point
RNA	Ribonucleic Acid
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
s	Second
SDS	Sodium Dodecyl Sulfate
SG	Sequence Group
ST	Sequence type
TAE	Tris-Acetic Acid-EDTA Buffer
TBE	Tris-Boric Acid-EDTA Buffer
TE	Tris-EDTA
UV	Ultraviolet light
VAP	Ventilator associated pneumonia

Chapter 1 INTRODUCTION

"Read not to contradict and confute; nor to believe and take for granted;
nor to find talk and discourse; but to weigh and consider."
Sir Francis Bacon
(1561-1626)

CHAPTER 1 – INTRODUCTION

1.1 The genus *Acinetobacter*

1.1.1 Taxonomy and Historical Perspective

The first description of an organism belonging to the genus *Acinetobacter* occurred in 1911 when the Dutch microbiologist and botanist Martinus Beijerinck isolated *Micrococcus calcoaceticus* from the soil using a calcium acetate-containing minimal medium (13). Several taxonomic modifications occurred since then. In 1954 Brisou and Prévot proposed the current genus designation, *Acinetobacter* (from the Greek ακινετσ [akinetos], which means nonmotile) (27), and in 1971 the Subcommittee on the Taxonomy of Moraxella and Allied Bacteria recommended that the genus *Acinetobacter* should include only oxidase-negative strains based on several studies of Paul Baumann (11, 12). The genus *Acinetobacter* is now included in the family *Moraxellaceae*; Order *Pseudomonadales*; Class *Gammaproteobacteria*; and Phylum *Proteobacteria*; comprising gram negative rods, with a DNA G/C content of 39 to 47 mol%, that are strictly aerobic, nonmotile, nonfastidious, nonfermentative, catalase positive, and oxidase negative (204).

During the 1970's *Acinetobacter* genus identification was established with the transformation assay of Juni (125), a method based on the property of the mutant *Acinetobacter* strain BD413 *trpE27*, recently identified as *A. baylyi* (34), to be competent for genetic transformation. A species was identified as belonging to the genus *Acinetobacter* when its crude DNA was able to transform the mutant strain to the wild-type phenotype. A decade after, the division of *Acinetobacter* isolates into genomic species become based on DNA-DNA reassociation studies and comprehensive phenotypic analysis, allowing the description of few DNA (hybridization) groups or genomospecies (25) (26, 194, 245). Currently the genus comprises 42 genomospecies, 33 with formal names (Table 1) and taxonomic characterizations are performed using several molecular methods, namely, amplified fragment length polymorphism (AFLP) (67), amplified rDNA restriction analysis (ARDRA) (259) or tDNA intergenic length polymorphism analysis (75). However, some of them are only available in reference laboratories.

The nucleotide sequence analysis of 16S rRNA and *rpoB* (RNA polymerase β-subunit) genes may be used as simple and accurate methods for *Acinetobacter* species identification, particularly the *rpoB* that exists in only one copy per cell, presents a high intraspecies similarity, and a small intragenic fragment of this gene (ca. 350 bp) displays a discriminatory power comparable to the one obtained with the complete 16S rRNA gene

sequence (136). In what concerns the most clinically relevant species belonging to *Acinetobacter* genus, *Acinetobacter baumannii*, identification has also been performed by the detection of the *bla*$_{\text{OXA-51-like}}$ gene which encodes for a species-specific carbapenemase. (29, 85, 86). However, the detection of non-*baumannii Acinetobacter* species harbouring *bla*$_{\text{OXA-51-like}}$-carrying plasmids revealed a possible drawback when identification is accomplished exclusively by this method (142). The increasing use of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS as a method for *Acinetobacter* spp. identification is now revealing promising results, including the discrimination among the species belonging to the so called *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex: *A. baumannii*, *Acinetobacter pittii* (formerly *Acinetobacter* genomic species 3), *Acinetobacter nosocomialis* (formerly *Acinetobacter* genomic species 13TU) and *Acinetobacter calcoaceticus* (189). They are closely related and difficult to distinguish from each other by phenotypic properties, making unsuitable many manual and semi-automated systems currently used for identification in routine clinical laboratories. However, since these species display different characteristics regarding colonization of human skin, antimicrobial susceptibility and mortality rates, a precise identification will orientate the therapeutic and improve the clinical outcome (84).

Table 1. Delineation of *Acinetobacter* genomic species [adapted from Peleg *et al*, 2008 (204)].

Species	Genomic species[a]	Type Strain	References
A. antiviralis		KNF2022T; KCTC0699BPT	(139)
A. baumannii	2	CIP 70.34; ATCC 19606T; DSM 30007	(25, 245)
A. baylyi		B2; CIP 107474; DSM 14961T	(34)
A. beijerinckii		NIPH 838T; LUH 4759T; CCUG 51249T; CCM 7266T; 58aT	(190)
A. bereziniae	10	LMG 1003T; CIP 70.12T; ATCC 17924T	(191)
A. bouvetii		DSM 14964T	(34)
A. brisouii		5YN5-8T; KACC 11602T; DSM 18516T	(5)
A. calcoaceticus	1	ATCC 23055T	(245)
A. gerneri		DSM 14968T	(34)
A. grimontii[b]*/ A. junii*	5	DSM 14967T; ATCC 17908T	(245)
A. guillouiae	11	LMG 988T; CIP 63.46T; ATCC 11171T; CCUG 2491T	(191)
A. gyllenbergii		NIPH 2150T; RUH 422T; CCUG 51248T; CCM 7267T; 1271T	(190)
A. haemolyticus	4	ATCC 17906T	(245)
A. indicus		DSM 25388T; CCM 7832T	(162)
A. johnsonii	7	ATCC 17909T	(245)
A. kyonggiensis		KSL5401-037T; JCM 17071T; KEMC 5401-037T	(138)
A. lwoffii	8/9	ATCC 15309T; ATCC 9957	(245)
A. marinus		SW-3T; KCTC 12259T; DSM 16312T	(277)
A. nosocomialis	13TU	LMG 10619T; CCM 7791T	(189)
A. oleivorans		DR1T; KCTC 23045T; JCM 16667T	(127)
A. parvus		NIPH384T	(186)
A. pittii	3	LMG 1035T; CIP 70.29T	(189)
A. radioresistens	12	IAM 13186T	(195)
A. rhizosphaerae		BIHB723	(105)
A. rudis		G30T; LMG 26107T; CCUG 57889T; DSM 24031T; CECT 7818T	(262)
A. schindleri		NIPH1034T	(185)
A. seohaensis		SW 100T; KCTC 12260T; DSM 16313T	(277)
A. soli		B1T; KCTC 22184T; JCM 15062T	(132, 205)
A. tandoii		DSM 14970T	(34)
A. tjernbergiae		DSM 14971T	(34)
A. towneri		DSM 14962T	(34)
A. ursingii, A. septicus[c]		NIPH137T; CCUG 56015	(185)
A. venetianus[d]		ATCC 31012	(260)
	6	ATCC 17979	(245)
	13BJ, 14TU	ATCC 17905	(25)
	14BJ	CCUG 14816	(26)
	15BJ	SEIP23.78	(26)
	15TU	M151a	(25)
	16	ATCC 17988	(26)
	17	SEIP Ac87.314	(26)
	Between 1 and 3	10095	(99)
	Close to 13TU	10090	(99)

[a] The genomic species according to Bouvet and Grimont classification (25). BJ refers to species delineation of Bouvet and Jeanjean (26) and TU refers to Tjernberg and Ursing (245); [b] *Acinetobacter grimontii* was recently reclassified as a later synonym of *Acinetobacter junii* (258); [c] There is a lack of evidence that "*Acinetobacter septicus*" is a different species from *Acinetobacter ursingii* (192); [d] The name *"Acinetobacter venetianus"* has been previously used to designate three marine hydrocarbon-degrading *Acinetobacter* strains, of which strain RAG-1 (ATCC 31012) has industrial applications for the production of the bioemulsifier emulsan. However, *A. venetianus* remains as a provisional designation awaiting further investigation (260).

1.1.2 Clinical relevance of *Acinetobacter* spp.

During many years, organisms belonging to the genus *Acinetobacter* were considered saprophytes of little clinical significance (77). This pattern started to change in the early 1970s with the recognition of the genus *Acinetobacter* as a significant nosocomial pathogen and more recently with the increasing frequency of drug-resistant related community and hospital acquired infections. This was probably a consequence of the introduction of powerful new antibiotics and the use of invasive diagnostic and therapeutic procedures in the clinical practice. (77, 249) In 2007, in one day survey along 75 countries aiming to provide information regarding the extent and patterns of infection in intensive care units (ICUs) *Acinetobacter* species were involved in 9% of all infections, highlighting the challenge that these pathogens present today (266).

In addition, community-acquired infection and infections acquired following war or natural disasters (e.g., earthquakes and tsunamis) have also been described. (64, 156, 197, 271) Nowadays we still have a debate regarding the clinical impact and mortality attributable to *Acinetobacter* spp.. The reasons for that discussion include the difficulty in distinguish between colonization and infection and in some studies the methods used for species identification were not appropriate according to current standards. (101, 204)

A. baumannii is the main genomic species associated with outbreaks of nosocomial infection. However it is of note the fact that in recent years we have assisted to increasing reports of non-*baumannii Acinetobacter* species associated with colonization and infection (14), namely *A. pittii*, *Acinetobacter lwoffii*, *Acinetobacter ursingii*, *Acinetobacter haemolyticus*, *A. calcoaceticus*, *A. nosocomialis*, *Acinetobacter johnsonnii* and *Acinetobacter junii* (47). Other studies refer the possibility that species like *A. ursingii*, *Acinetobacter schindleri* or *Acinetobacter bereziniae* might be underestimated due to their absence in the databases of all commercial biochemical kits (135). There are two reports of *Acinetobacter baylyi* isolated from clinical samples, although only one refers the species as the agent of infection (40). The detection of these species might be explained by the use of more accurate methods to routinely perform species identification (256).

1.1.2.1 Infections caused by *A. baumannii*

Hospital acquired *A. baumannii* infections include ventilator-associated pneumonia (VAP), skin and soft tissue infections, wound infections, urinary tract infections, secondary meningitis, and bloodstream infections (68). In a 2006-2007 antimicrobial-resistant pathogens associated with healthcare-associated infections survey (United States of America-USA), *A. baumannii* was the third more frequent species associated with VAP,

and the ninth most frequent species implicated in central line-associated bloodstream infections, catheter-associated urinary tract infections and surgical site infection (113). The species' ability to acquire antimicrobial resistance determinants and the capacity to survive for long periods on dry surfaces (267, 268) contribute for a long persistence in the hospital setting. *A. baumannii* infections concern particularly ill patients in ICUs, mainly those requiring mechanical ventilation, and patients with wound or burn injuries (249). The factors facilitating colonization or infections include increased length of hospital stay (although in outbreak situations may occur an earlier acquisition of infection), previous antibiotic therapy, mechanical ventilation, use of catheters, sutures, dialysis, exposure to patients colonized or infected with *A. baumannii*, environmental contamination, understaffing and poor adherence of staff to hand hygiene (174, 249).

A. baumannii infections in the community setting have been mainly reported from countries with tropical or subtropical climate, probably due to the preference of the genus for moisture (6). These infections usually affect patients with some form of comorbidity like chronic obstructive pulmonary disease, renal disease, and diabetes mellitus or associated with heavy smoking and excess alcohol consumption, which may facilitate aspiration of pharyngeal bacteria. The community-acquired infections might include pneumonia (in the majority of cases), meningitis, soft-tissue infection, ocular infection, urinary tract infection, and native valve endocarditis. (6, 87, 119, 238)

In recent years *A. baumannii* was increasingly isolated from wounds of combat casualties from Iraq and Afghanistan, has already occurred during the Vietnam War, identified at that time as *Mimeae-Herellea-Bacterium-Alcaligenes*) (36, 64, 248). Although was raised the possibility that inoculation could occur at the time of injury, both from previously colonized skin or contaminated soil, posterior studies concluded that *A. baumannii* acquisition occurred at health care facilities (232).

Finally, the association of *A. baumannii* infection with natural disasters was evidenced in Marmara earthquake, Turkey, 1999, where *A. baumannii* was the most frequently isolated species among the trauma victims, especially from wound infections (197). After the earthquake in Wenchuan, China in 2008 *A. baumannii* was also the most frequently found in wound and sputum, and the third pathogen recovered from blood among the victims. (271)

1.1.2.2 Infections caused by non-*baumannii Acinetobacter* species

While *A. baumannii*, frequently associated with outbreaks, *A. pittii* and *A. nosocomialis* are the most common of the *Acinetobacter* species among clinical isolates, *A. johnsonii*, *A*

junii, *A. lwoffii*, *Acinetobacter parvus*, *Acinetobacter radioresistens*, *A. schindleri* and *A. ursingii* became more frequently associated with infection, particularly, catheter-related bloodstream infections (68, 253, 256). *A. haemolyticus*, rarely identified in clinical isolates, although associated in our country with carbapenem resistance (215), has been implicated in cerebrospinal meningitis (102) or prosthetic infective endocarditis (35). *A. lwoffii,* a human commensal frequently found colonizing the skin of healthy persons, has been increasingly associated with nosocomial infections (68). Additionally, has been linked to acute gastritis, very similar to the one caused by *Helicobacter pylori* (217) and to pneumonia, including in the community setting (176).

1.1.3 *Acinetobacter* spp. ecology

1.1.3.1 Ecology of *A. baumannii*

Species belonging to *Acinetobacter* genus have been recovered from soil, water and animals (68, 249). *A. baumannii* seems to be a rare colonizer of human skin in temperate climates (234), although common in tropical environments (49). This species has been identified in vegetables (16), with isolates presenting high rates of resistance to ciprofloxacin and gentamicin, findings that raised the question of being the food one of this species sources in the hospital setting. *A. baumannii* was also identified in aquacultures of fish and shrimp farms although was not determined if it constituted an environmental niche or was due to human contact (121). *A. baumannii* detection in hospitalized dogs, cats and horses raises concerns about the possibility of spread between humans and animals (82, 94, 284). The presence of this species in 22% sampled body lice of homeless persons raises the possibility that louse might constitute an *A. baumannii* reservoir and vector of transmission, although was speculated that this detection could also be due to undiagnosed transient *A. baumannii* bacteremia or even contamination (137).

With the increasing reports of *A. baumannii* outside the hospital setting the discussion concerning the origin of the organism revived. However, the existing data is not sufficient to accurately define *A. baumannii* natural habitat, but it appears to be neither commensal nor a typical environmental organism (204).

1.1.3.2 Ecology of non-*baumannii Acinetobacter* species

Several *Acinetobacter* species are normal colonizers of human skin, particularly *A. lwoffii*, *A. johnsonii*, *A. junii*, *A. pitti*, *A. radioresistens*, and *A. haemolyticus*, although their frequency may vary according to the season, region, and may increase in hospitalized patients (15, 202, 234). *A. calcoaceticus*, *A. pittii*, *A. johnsonii, A. lwoffii* and *Acinetobacter*

genomic species 11 have also been found in water, soil and vegetables (119), and *A. johnsonii* was also associated with the human and fish intestinal tract (69, 104). In addition, *A. haemolyticus* and *A. lwoffii* have been detected in aquacultures environment and associated with fish intestinal content (104). Besides these species, which may be considered as opportunistic pathogens, other species have utility for biotechnology applications (242).

1.1.4 Treatment

Until 1970s, treatment of *Acinetobacter* infections included several therapeutic options, such as aminoglycosides, β-lactams and tetracyclines (14). Since then, *Acinetobacter,* and in particular, *A. baumannii*´s remarkable ability to accumulate a myriad of resistance mechanisms has limited the therapeutic options (201).

Carbapenems are β-lactam antimicrobial agents introduced in therapeutics in 1985 and presenting an exceptionally broad spectrum of activity. Carbapenems are stable to most β-lactamases including AmpC β-lactamases and extended-spectrum β-lactamases (ESBLs) and have long been regarded as the agents of choice for the treatment of infections caused by *Acinetobacter* spp., but resistance rates have risen substantially in some areas, with susceptibility to the carbapenems ranging from 90% to as low as 32%, depending on the geographic region and the carbapenem tested (91, 281).

β-Lactamase inhibitors, particularly sulbactam, revealed intrinsic activity against many *Acinetobacter* strains, with a bacteriostatic or bactericidal mechanism of activity, depending on the strain examined, and mediated via penicillin binding proteins (PBPs). Sulbactam was used, particularly in combination with rifampin, colistin or fosfomycin, during the last decade for the treatment of carbapenem-resistant *Acinetobacter* spp. (130, 163, 198). However, the high level of resistance occurring nowadays to sulbactam hampers its use against MDR *A. baumannii* (223). Polymyxins are cationic polypeptides discovered in 1947 from different species of *Bacillus polymyxa* (122). Their mode of activity is the interaction with the lipopolysaccharide layer of Gram-negative bacteria. In the early 1980s polymyxins were withdrawn from clinical use due to toxicity reports, namely neurotoxicity and nefrotoxicity, being used only for the treatment of cystic fibrosis. However, the emergence of Gram negative bacteria resistant to almost all classes of available antibiotics except polymyxins contributed for their revival. This class of antibiotics consists of five chemically different compounds, polymyxin A, B, C, D, and E (colistin) but only two are currently available for clinical use - namely, polymyxin B and colistin (polymyxin E). Polymyxins show bactericidal activity against *A. baumannii*, and

resistance rates against these agents have remained low, even in multidrug-resistant and carbapenem-resistant isolates(223). However, there are recent reports of heteroresistance among *A. baumannii* isolates, resulting from the existence of subpopulations of resistant *A. baumannii* within colistin-susceptible clinical isolates, particularly in patients with prior colistin therapy (110). These findings raise the concern about the use of colistin, especially in monotherapy, as a therapeutic option in the treatment of MDR *A. baumannii*.

Aminoglycosides constitute one of the oldest classes of antimicrobials, presenting activity by binding to 16S rRNA in the 30S ribosomal subunits and inhibiting protein synthesis (Magnet and Blanchard, 2005). Regardless the nephro- and ototoxicity, aminoglycosides, in particular amikacin and tobramycin, retain good activity against MDR *Acinetobacter* spp. (199). However, resistance to these agents is also increasing, being recommended their use in combination with other antimicrobials. Inhaled tobramycin has shown good activity in association with an intravenous β-lactam in the treatment of VAP (107) and intrathecal amikacin in combination with colistin has been effective in the treatment of meningitis (96).

Fluoroquinolones are broad-spectrum bactericidal agents used to treat diverse bacterial infections and levofloxacin, moxifloxacin and ciprofloxacin previously presented good activity against *Acinetobacter* spp. (111, 153). New derivatives are now under pre-clinical studies, in order to overcome the increasing resistance observed among this genus, and in particular *A. baumannii* (153).

Glycylcyclines are a novel class of antimicrobial agents related to the tetracyclines presenting a similar mechanism of action. They are represented by tigecycline, a semisynthetic derivative of minocycline, which was licensed for the treatment of complicated skin and soft tissue infections (261), and intra-abdominal infections (196) and has shown a good *in vitro* activity against *A. baumannii*. However, several studies indicate that tigecycline may not be consistently active against the multidrug-resistant isolates (184, 200) and the reports of resistance development in the course of the treatment (118) suggest that should only be used in combined regimens with other antimicrobials, such as levofloxacin, amikacin, imipenem, and colistin (213).

Rifampicin has demonstrated *in vitro* and *in vivo* bactericidal activities against multi-drug resistant (MDR) *A. baumannii*, including severe infections caused by imipenem-resistant strains (143). However, the easily development of resistance, due to RNA polymerase mutations, emphasizes the need to be associated with other antibiotics, generally colistin (10).

Azithromycin has been tested in the treatment of *Acinetobacter* spp. infections, generally in combination with other antimicrobials like colistin, meropenem and colistin, showing synergistic effects (244).

Tetracyclines constitute another option for *Acinetobacter* spp. infections treatment. From a recent work concerning ventilator acquired pneumonia (VAP) due to carbapenem-resistant *A. baumannii* was concluded that VAP could be effectively treated with minocycline or minocycline/ doxycycline (37, 272).

Although clinical data are lacking, combination therapy seems to be the better option for the treatment of patients with multidrug-resistant *A baumannii* with *in vitro* and animal studies suggesting the use of colistin plus rifampin or azithromycin, imipenem or azithromycin plus rifampicin and the triple combination of imipenem, rifampicin and colistin (134, 145).

1.1.5 Antimicrobial Resistance

Acinetobacter spp. exhibit a natural reduced susceptibility to antibiotics explained by the low permeability of the outer membrane, the constitutive expression of some efflux pumps or the interplay between the two processes. In addition, several species present intrinsic β-lactamases (263). The increasing antimicrobial resistance displayed by these organisms, and in particular *A. baumannii*, has largely contributed for their persistence in the hospital setting limiting the therapeutic options. In addition, the recent reports of *A. baumannii* strains resistant to all available antibiotics, whether called pandrug, extreme or extensively drug resistant, according with the currently accepted terminology (Table 2), raise concerns about returning to the pre-antibiotic era (73, 157, 200).

Table 2 - Definitions regarding antimicrobial resistance, proposed by Magiorakos *et al*, 2012 **(157)**

MDR ***Multidrug Resistant***	Acquired non-susceptibility to at least one agent in three or more antimicrobial categories.
XDR ***Extreme Drug Resistant***	Non-susceptibility to at least one agent in all but two or fewer antimicrobial categories.
PDR **Pandrug-Resistant**	Non-susceptibility to all agents in all antimicrobial categories.

1.1.5.1 Resistance to β –lactams

β -lactams constitute the most flexible antibiotic class, with a great versatility and diversity in what concerns chemical properties, antibacterial spectra and administration plans. Resistance to β-lactams in *Acinetobacter* spp. can occur due to enzymatic (β-lactamases)

or nonenzymatic mechanisms (loss of or decrease in the expression of porines; increased expression of efflux pumps or penicillin binding proteins – PBPs - alterations).

1.1.5.1.1 β –lactamases

The β-lactamases are divided into A-D four classes based on a molecular classification (4). Classes A, C and D belong to serine type enzymes with a serine moiety at the active site, whereas class B enzymes require divalent metal cations as cofactors (eg. Zn^{2+}) being designated metallo-β-lactamases (MBL) (269).

A. baumannii possesses two intrinsic β-lactamases: a class C chromosomally encoded cephalosporinase (ADC, for *Acinetobacter* derived cephalosporinase) and a class D oxacillinase (OXA-51-like)(225, 255). Other *Acinetobacter* species like *A. haemolyticus*, *A. lwoffii*, *A. johnsonii*, *A. radioresistens* and *A. calcoaceticus* also present naturally occurring CHDLS, OXA-214-like, OXA-134-like, OXA-211-like, OXA-23-like and OXA-213-like, respectively (89).

ADCs are normally expressed at low levels and are not inducible (23, 120). However, both ADCs and OXA-51 expression levels might be augmented by the presence of insertion sequences (224, 254, 255, 263). The role of OXA-51 will be discussed later on, since it has become an important tool for epidemiological studies and genetic marker for identification of *A. baumannii* species.

The acquired class A β-lactamases reported in *Acinetobacter* spp. include at least nine families: TEMs (83), SHVs (182), CARBs (212), CTX-Ms type (183), SCO (207), PERs(181), GES (235), VEBs (181) and KPCs (220) (Table 3). However, the β-lactamases that confer resistance or reduced susceptibility to carbapenems (carbapenemases), belonging to classes D, B and A (in order of frequency), are the ones that truly constitute a sentinel event for antimicrobial resistance and will be detailed in the next section.

1.1.5.1.1.1 Carbapenemases

Historically, carbapenems have been considered the best therapeutic response in infections caused by MDR *Acinetobacter* spp. and detection of carbapenemase-producing isolates always constitutes a concerning event.

The most disseminated carbapenemases belong to class D β-lactamases group (carbapenem-hydrolyzing class D β-lactamases - CHDLs).

The first report of an acquired class D β -lactamase with carbapenemase activity occurred in 1993 (206). The enzyme OXA-23, initially designated as ARI-1 (for *Acinetobacter*

resistant to imipenem), was detected in a multidrug-resistant *A. baumannii* clinical isolate collected in Scotland in 1985 (203). Few years later was demonstrated its plasmid location (231), although a chromosomal location is also common (177). In the following years were described closely related derivatives for this enzyme, constituting together the OXA-23 group which comprises today fifteen variants (Table 3). OXA-23 cluster displays 48% of amino acid homology with the OXA-58 cluster, 60% homology with OXA-40 cluster and 63% homology with OXA-143 cluster.

A second group of CHDLs corresponds to the OXA-24/OXA-40 group, which includes seven variants. The $bla_{\text{OXA-24/40}}$ gene was originally identified as chromosomally encoded in carbapenem-resistant *A. baumannii* isolates recovered in Spain and France (24, 112) but was later demonstrated its presence in plasmids of *A. baumannii* and *A. haemolyticus* clinical isolates from Portugal (215) and subsequently in Italy (169) and Spain (227) were OXA-24/40 was also found in *A. calcoaceticus* species. OXA-40 cluster displays 60% homology with OXA-23 cluster, 47% homology with OXA-58 cluster and 88% homology with OXA-143 cluster (115, 204).

A third group of CHDLs is represented by OXA-58 (208), including at this moment four variants (Table 3). The $bla_{\text{OXA-58}}$ gene was found to be either plasmid and/or chromosome encoded. OXA-58 cluster presents 48%, 47% and 52% of aminoacidic identity with OXA-23 cluster, OXA-40 cluster and OXA-143 cluster respectively (115, 204)

Finally, OXA-143, first identified in Brazil, encoded in a plasmid, represents a fourth group of acquired CHDLS, revealing 88% amino acid sequence identity with OXA-40, 63% identity with OXA-23, and 52% identity with OXA-58 (115).

CHDLs present hydrolytic activity against penicillins, extended-spectrum cephalosporins, methicillin, aztreonam and carbapenems(230). They are resistant to inhibition by clavulanate and tazobactam but most are susceptible to NaCl inhibition, which provides a means of their laboratory identification. The level of carbapenem-hydrolysis by CHDLs is considerably low, with imipenem being the preferred substrate over meropenem (214).

Class B β-lactamases or metallo-β-lactamases (MBLs) are able to hydrolyze carbapenems, cephalosporins and penicillins but lack the ability to hydrolyze aztreonam and are resistant to the clinically available β-lactamase inhibitors. Although MBLs are much more potent in carbapenem-hydrolyzing activity (100 to 1000-fold higher) than CHDLs (204), they are described with less frequency in *A. baumannii*. MBLs families described until now in *A. baumannii* include the IMP, VIM, SIM, and more recently SPM and NDM (Table 4). IMP-type MBLs were initially described in *Pseudomonas aeruginosa* isolates from Japan in 1990 (209). Until now, were identified thirty three IMP variants, from

which eight were detected in *A. baumannii* (Table 3) VIM-type MBLs, whose five variants were identified in *A. baumannii* (Table 3), were initially described in Italy and generally present a higher affinity toward carbapenems in comparison to IMP enzymes (71). SIM-1 was identified in a Korean isolate, and that remains the only description of SIM enzymes in *A. baumannii* (140). SPM has also a low frequency in *A. baumannii*, with only one description (SPM-1) from Iranian clinical isolates (235). Finally, the novel class B MBL enzyme, NDM, initially reported in *Klebsiella pneumoniae* and *Escherichia coli* clinical isolates, has increasingly been reported in *Acinetobacter* spp, particularly in *A. baumannii* and *A. lwoffii,* with two variants described until now (NDM-1 and NDM-2) (Table 3) (20, 100).

In what concerns CHDLs global distribution, OXA-23 is the most widespread, being frequently detected in isolates from Asian countries, (eg. China, Korea)(276, 282), but also reported in South America in countries such as Brazil, Colombia (62, 265) and particularly in Europe (58, 128, 177) (Figure 1). The OXA-58 is frequently found in Europe (8, 168). OXA-24/OXA-40 is not so disseminated as OXA-23 but has been identified in different areas, especially in Portugal, Spain, France and United States (24, 57, 61, 150, 152, 169, 215)(Figure 1). In Asia, an OXA-24/OXA-40 derivative, the OXA-72 was also detected with some frequency (154), and more recently in isolates from Croatia and USA (243, 273, 280) (Figure 1). OXA-143 has only been described in Brazil (115, 274).

In comparison with acquired CHDLs, which have been mostly identified in *A. baumannii,* MBLs have been identified in a wide variety of Gram-negative species, but less frequently in *A. baumannii* (52, 209) (Figure 2). Only the recently described NDM (variants NDM-1 and NDM-2) seem to have a more wide dissemination among *Acinetobacter* species (39, 41, 42, 100, 126, 129, 133, 144).

In Portugal, the prevalence of carbapenem-resistant *A. baumannii* has increased since 1998 (59, 61, 215). Previous studies identified an OXA-40 producing clone as endemic in our country, constituting a sub-cluster of EUII (59). Besides this dominant clone, two other clones defined by *Apa*I-pulsed-field gel electrophoresis (PFGE) typing, were identified among the carbapenem resistant *A. baumannii* population but occurring with a less frequency (215). Additionally, the description of OXA-40 in a different *Acinetobacter* species, *A. haemolyticus,* raised the possibility that horizontal dissemination of the $bla_{\text{OXA-40}}$ gene could contribute for its occurrence among different species (215).

Finally, in Portugal the only MBL described in *A. baumannii* was IMP-5, an enzyme also identified in *Pseudomonas aeruginosa* isolates (28, 60). This enzyme genetic vicinity was

recently characterized being detected its association with a Tn*402*-like transposon (278). These findings raise the possibility of dissemination, not only among *A. baumannii*, but also between different species.

Figures 1 and 2 present the main occurrences of CHDL enzymes and MBL enzymes, respectively, in *Acinetobacter* spp. clinical isolates at a global level.

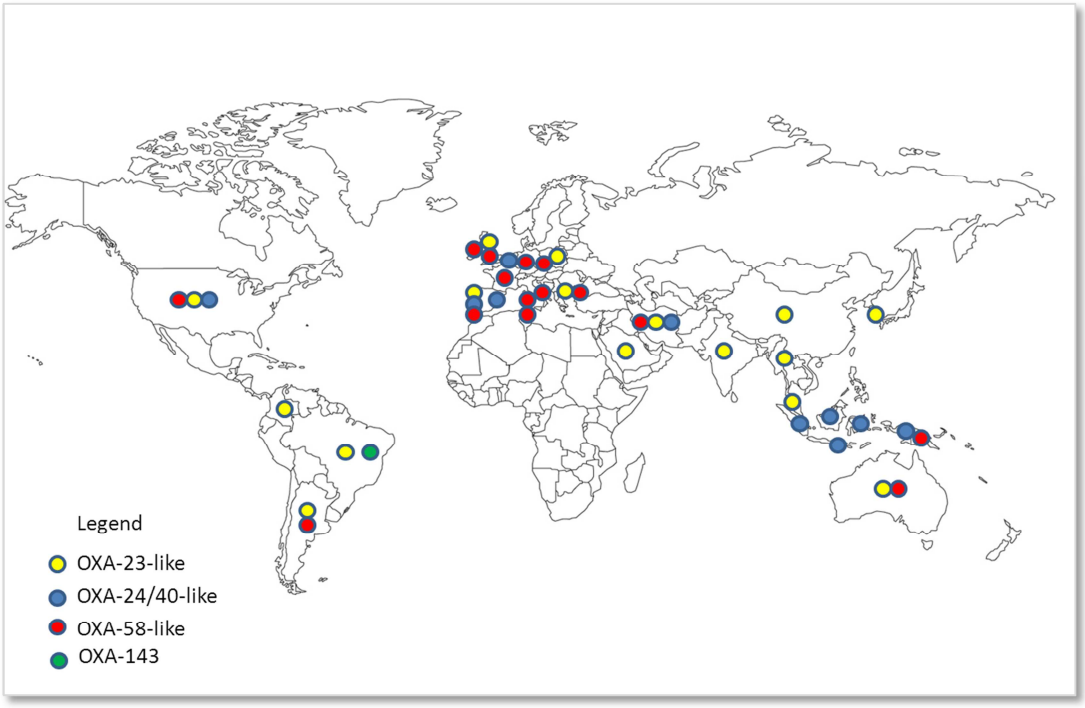

Figure 1. Global distribution of CHDLs enzymes reported in *Acinetobacter* spp.

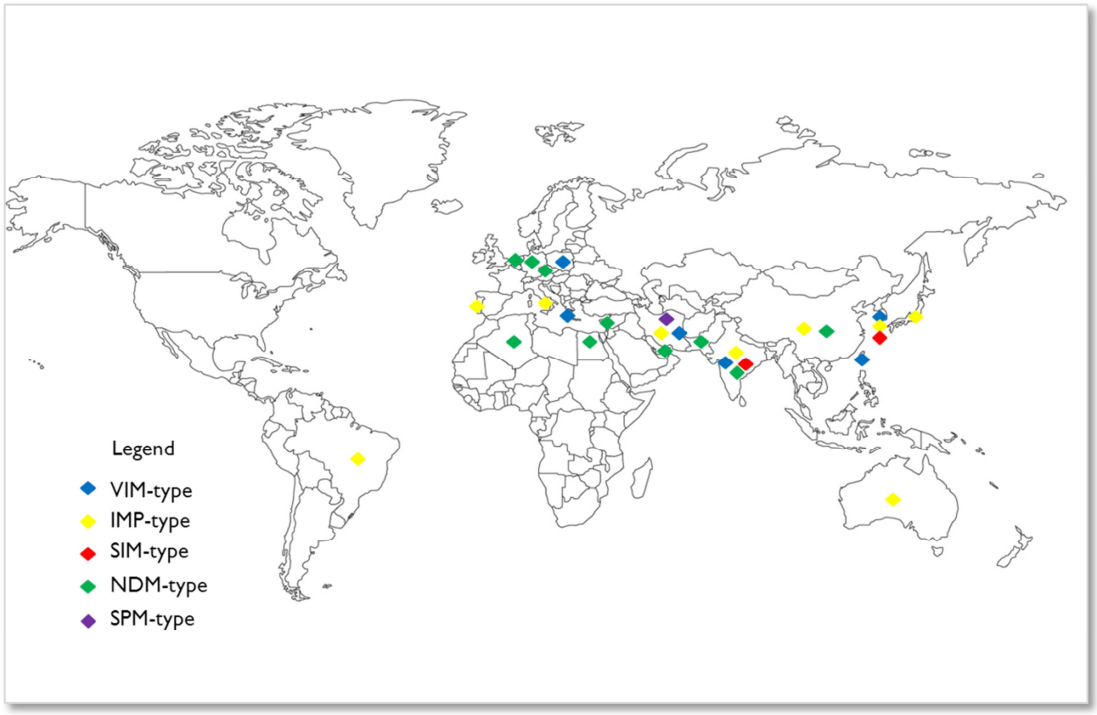

Figure 2. Global distribution of MBLs enzymes reported in *Acinetobacter* spp.

Class A carbapenemases are relatively rare in *Acinetobacter* spp., being described in *A. baumannii* some members of the families GES (GES-14) detected in France and Belgium (19), and KPC (KPC-2, -3, -4 and -10) in Puerto Rico (220, 221)(Table 3), being able to hydrolyze a broad variety of β-lactams, including carbapenems, penicillins, cephalosporins and aztreonam (in the case of KPC), but can be inhibited by clinically available β-lactamase inhibitors such as clavulanate and tazobactam (19, 214, 220, 221, 235).

1.1.5.1.2 Alterations on membrane permeability

Non-enzymatic mechanisms such as alterations on membrane permeability (decrease or absence of porines, and overexpression of efflux pumps) and PBPs alterations, might also contribute for β-lactam resistance, including to carbapenems. When these alterations occur simultaneously with the production of β-lactamases usually are detected high carbapenem MICs (263).

1.1.5.1.2 .1 Porins

Porins (outer membrane proteins – OMPs) form channels that allow the transport of molecules across lipid bilayer membranes. *A. baumannii* reveals a decrease in outer membrane permeability (less than 5%) when compared with other Gram-negative

organisms which might be explain by the small number and size of porins (263). Additionally, changes in membrane permeability due to the loss of OMPs are usually implicated in resistance to β-lactams. The major OMP in *A. baumannii* is OmpA which plays an important role in bacterial pathogenesis, quorum sensing and biofilm formation, regulating adhesion to surfaces. It seems that is also implicated in the entry of β-lactams and saccharides up to approximately 800 Da and, therefore, the lack of this porin could contribute to high levels of resistance (263). Other Omps whose reduced expression has been associated with carbapenem resistance include the 33- to 36-kDa Omp, 47-, 44-, 37-kDa Omp and 43 KDa Omp (protein homologous to the OprD of *Pseudomonas aeruginosa*) (50, 79) (Table 3). Other studies have shown that the loss of a heat-modifiable 29-kDa OMP, designated CarO, was also responsible for imipenem resistance (146, 179, 180)(Table 3).

1.1.5.1.2 .2 Efflux systems

The main function of efflux systems is to remove chemicals that could be toxic for the bacterial cell. However, in the last years it has become patent that efflux pumps have a potent ability to actively eliminate several classes of antibiotics, including β-lactams. Until now were described six families of pumps: ATP-binding cassette (ABC) transporters, the small multidrug resistance (SMR) and multidrug and toxic compound extrusion (MATE) families, the major facilitator superfamily (MFS), the bacterial integral membrane proteins (BIMP), and the resistance-nodulation-cell division (RND) (263). RND systems are the most prevalent in *A. baumannii*, including the AdeABC, AdeIJK and AdeFGH efflux systems (where Ade stands for A*cinetobacter* drug efflux) (Figure 3). Among non-*baumannii Acinetobacter* species, AdeDE pump has been identified in *A. pittii* and *A. nosocomialis* (38, 48) and more recently was detected an AdeABC-like efflux system in *A. nosocomialis* (222). This family of efflux pumps expels the antimicrobial by utilizing the proton motive force as the driving force for efflux, exhibiting a wide substrate range that can include antibiotics, dyes, biocides, detergents, and antiseptics. Among the systems belonging to this family, overexpression of AdeABC constitutes a major mechanism of multiresistance in *A. baumannii,* including to β-lactams (164). The *adeABC* operon encodes the AdeA major fusion protein (MFP), the multidrug transporter AdeB (inner membrane protein – pump), and the AdeC outer membrane factor (OMF). Overexpression of this system (identified in ca. 80% *A. baumannii* strains) results from mutations in a two-component regulatory system encoded by the genes *adeR* (regulator gene) and *adeS* (sensor kinase) (164) (Figure 3). Although several studies indicate the propensity of *A. baumannii* to achieve multidrug resistance by overexpression of the efflux pump when exposed to its substrates, the signal recognized by AdeS, the mechanism of regulation of

AdeABC by AdeRS and the DNA-binding site of AdeR remain to be elucidated (116). AdeIJK is intrinsic of *A. baumannii* species and is responsible for resistance to various drug classes including β-lactams, such as ticarcillin, cephalosporins and aztreonam (155). Overexpression of this system above a certain level results in toxicity for the bacterial cell. Therefore, its transcription is tightly regulated by a TetR regulator encoded by the *adeN* gene (155). Figure 3 shows a schematic representation of the main RND efflux systems present in *A. baumannii* and responsible for antibiotic resistance.

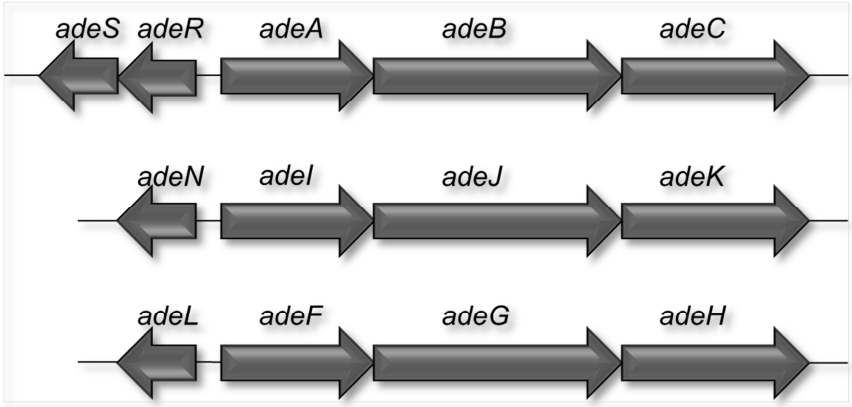

Figure 3 - Schematic representation of operons for the main efflux systems belonging to RND family in *A. baumannii*. Arrows represent coding sequences and indicate direction of transcription. adeS, adeR, adeN and adeL encode transcriptional regulators. [Adapted from Coyne *et al*, 2011 (55)].

1.1.5.1.3 Alterations of penicillin binding proteins (PBPs)

Resistance to β-lactams might also occur by changes in the affinity or expression levels of PBPs, although information regarding this mechanism is still limited (Table 3). However, in order to result in carbapenem resistance, these mechanisms usually occur associated with β-lactamases, overexpression of efflux pumps or decreased expression of OMPs (279). Until now were identified seven PBPs (PBP1, PBP1b, PBP2, PBP3, PBP5/6, PBP6b, PBP7/8) that might contribute to carbapenem resistant phenotype in *A. baumannii* (51). However, carbapenem resistance has only been associated with decreased expression of the PBP2 (88).

1.1.5.2 Resistance to aminoglycosides

Aminoglycosides have been used for a long time being an important alternative for therapy of infections caused by MDR strains. Resistance to aminoglycosides by aminoglycoside-modifying enzymes (AMEs) is also an important feature in the multidrug-

resistant phenotype of *Acinetobacter* spp. AMEs include acetyltransferases (74, 226), nucleotidyltransferases and phosphotransferases, with some *A. baumannii* strains often containing multiple enzymes of these classes (101). High level resistance to aminoglycosides occurs in the presence of another group of enzymes, the 16S rRNA methyltransferases, with the *armA* gene being relatively widespread in *A. baumannii* (43, 72, 283) (Table 3).

Overexpression of efflux pumps such as AdeABC and AbeM (MATE family) also contributes for aminoglycoside resistance (263) (Table 3).

1.1.5.3 Resistance to tetracyclines and glycylcyclines

Resistance to tetracyclines and glycycyclines (tigecycline) may occur by overexpression of RND efflux systems (AdeABC, AdeFGH and AdeIJK) (56, 63, 117, 147, 155). Resistance to tetracyclines may also implicate efflux systems of the MFS family (TetA, TetB) and might also involve the *tetM* gene, associated with tetracycline ribosomal protection (3, 55).

1.1.5.4 Resistance to colistin

Resistance to colistin is associated with mutations in the first three genes of the lipid A biosynthesis pathway, *lpxA*, *lpxC*, and *lpxD*, which results in the complete loss of LPS production. This results in the escape to the first step of colistin action which is the electrostatic interaction between the positively charged antibiotic molecule and the negatively charged lipid A (component of lipopolysaccharide - LPS) (172, 173, 239). Additionally, mutations in *pmrA* and *pmrB* may be linked to colistin resistance being responsible by the removal of an initial charge-based interaction between the bacterial outer membrane lipid bilayer and colistin (2).

1.1.5.5 Other resistance mechanisms

Mechanisms of fluoroquinolone resistance in *A. baumannii* include mutations in *gyrA* and *parC* encoding for DNA gyrase and DNA topoisomerase IV, respectively (148). Finally, trimethoprim resistance seems to involve mutations in a putative dihydrofolate reductase gene (*folA*) (161).

Table 3 summarizes the major mechanisms of resistance that have been identified for the different classes of antibiotics in *Acinetobacter* spp.

Table 3. Major antimicrobial resistance mechanisms in *Acinetobacter* spp.

Antimicrobial Class	Resistance Mechanism	Class/family	Variants	Reference
β -lactams	β-lactamases	Class C Intrinsic cephalosporinases	AmpC (ADC-1 to -4, -6, -7, -10, -11, -25, -26, -29 to -33,-38, -39, -50 to -54, -56)	(120) (19, 21, 83, 182, 183,
		Class A	TEM-1, -2 TEM-type ESBLs: TEM-92, -116, -128, -150 SHV-1b,-2, -5, 12, -18, -56, -71, -96 CARB-2, -4, -5, -8, -10 VEB-1, -1a, -3 PER-1, -2, -7 CTX-M-2, -5, -15, -43 SCO-1 GES-1, -11, -12	212, 240) (19, 220) (20, 126, 209, 235) (89, 115, 209) (50, 103, 146)
		Carbapenemases		(98, 158)
		Class A	KPC-2, -3, -4, 10 GES-14	
		Class B – MBLs	IMP-1, -2, -4, -5, -6, -8, -11, -14, -19 VIM-1, -2, -3, -4, -11 SIM-1 SPM-1 NDM-1, -2	
		Class D – CHDLs	OXA-23 group (OXA-23, -27, -49, -102, -103, -105, -133, -146, -165 to -171) OXA-24/40 group (OXA-24/40; -25, -26, -72, -139, -160, -182) OXA-58 group (-96, -97, -164) OXA-143 [a]OXA-51 group; OXA-214-like, OXA-134 group, OXA-211group, OXA-213-group	
	OMP alterations	CarO OmpA 33-36 KDa protein 43 KDa protein		
	Efflux pump	RND	AdeABC	
	Altered PBP expression		PBP2 downregulation	

Table 3. Major antimicrobial resistance mechanisms in *Acinetobacter* spp. (cont.)

Antimicrobial Class	Resistance Mechanism	Class/family	Variants	Reference
Tetracyclines	Efflux pump	MFS RND	TetA, TetB AdeABC, AdeFGH, AdeIJK	(263)
	Ribosomal protection		TetM	(218)
Glycylcyclines	Efflux pump	RND	AdeABC, AdeFGH, AdeIJK	(229)
Aminoglycosides	Enzymatic degradation	Acetyltransferases Nucleotidyl-transferases Phospho-transferases	AacC1/2, AadA, AAdB Ant1 AphA1, AphA6	(187)
	Efflux pumps	RND MATE	AdeABC AdeM	(263)
	16S rDNA methytransferases		ArmA	(72)
Quinolones	DNA gyrase mutations Topoisomerase mutations	GyrA ParC		(108)
	Efflux pumps	RND MATE BIMP	AdeABC AdeM AbeS	(263)
Chloramphenicol	Efflux pumps	RND MFS BIMP	AdeABC AdeIJK CmlA CraA AbeS	(263)
Trimethoprim/ sulfamethoxazole	Efflux pump	RND	AdeABC, AdeIJUK	(263)
	Dihydropteroate synthase		SulI/II	(101)
	Dihydrofolate reductase		FolA	(161)
Macrolides	Efflux pumps	MATE BIMP	AbeM AbeS	(263)
Polymyxins	PmrAB two component mutation signalling proteins		PmrA and PmrB	(2)
	Loss of the lipid A component of lipopolysaccharide		LpxA, LpxC, LpxD	(173)

1.2 Resistance mobilization

1.2.1 Mobile Genetic Elements

Acinetobacter spp. display a remarkable capacity to acquire and disseminate resistance mechanisms, a feature associated with specific genetic elements such as insertion sequences, transposons, integrons (not mobile by themselves), plasmids and resistance islands (223).

1.2.1.1 Insertion sequences and composite transposons

Insertion sequences (IS) are defined as short mobile DNA elements, up to 2 kbp, and are the most abundant transposable elements (206). IS are constituted by one or two ORF encoding a transposase, the enzyme that catalyzes the transposition of the IS element. The transposase is usually surrounded by short terminal repeats and insertion occurs by duplication of a direct repeat, a short sequence of the target DNA flanking the IS (159, 160). Miniature inverted-repeat transposable elements (MITEs) are nonautonomous ISs deleted for part or all of the transposase ORF but retaining both ends (223, 237). It is important to note that besides acquisition of accessory functions ISs are also involved in other events, such as chromosome rearrangements and plasmid integration (159, 160)

Many IS elements present promoter hexamers located in the terminal IRs (−35) that when are placed (by transposition) at the correct distance from a resident −10 hexamer, new promoters are able to activate the expression of neighboring genes. Probably this is the reason why CHDLs genes are usually associated with ISs, since they present a poor catalytic activity against carbapenems and need expression enhancers. Among the ISs that have been reported in *Acinetobacter* spp (more than 30 different types) (237). IS*AbaI* and IS*Aba125* seem to be the most common. IS*Aba1* is frequently found adjacent to *ampCs* and bla_{OXAs} as well as adjacent to *sulII* (178). Curiously, was identified in *Acinetobacter* spp., but not in *Enterobacteriacea* and *Pseudomonas aeruginosa*, being suggested that this IS is customized for *Acinetobacter* (233).

Additionally, IS*AbaI*, IS*Aba2*, IS*Aba3*, IS*Aba4*, IS*Aba10*, IS*Aba16*, IS*Aba125*, IS*Aba825*, and IS*18* were found upstream from bla_{OXA-23} and bla_{OXA-58} (17, 53, 151, 209, 210, 216). bla_{OXA-58} usually appears associated with IS*Aba1*, IS*Aba2*, IS*Aba3*-like and, and IS*18* (210). The bla_{OXA-23} gene may be mobilized through different genetic structures, such as the composite transposon Tn*2006*, formed by two IS*Aba1* elements. IS*Aba4* was also found upstream of the bla_{OXA-23} gene but in a single copy, constituting the Tn*2007* (178). Finally, IS*Aba9* has been described upstream from both bla_{OXA-51} and the gene encoding CARB-10 (90, 212).

IS elements may also be associated with resistance by disrupting coding sequences of certain genes. For instance, the disruption of *carO* gene resulting in carbapenem resistance, or the *lpxA* or *lpxC* genes disruption leading to colistin resistance (141, 172).

Composite (or compound) transposons are structures in which a DNA segment is flanked by two copies of a given IS that act in concert, contributing for the mobilization of that region (159). An example of this structure is the already mentioned Tn*2006*, composed by two copies of IS*Aba1* and usually associated with bla_{OXA-23} (177). Another example is the In*76* encoding for the MBL IMP-5 which was found to be embedded in aTn*402*-like transposon. In this structure In*76* was flanked by MITE-like structures suggesting that a MITE-facilitated transposition-like mechanism is responsible for the mobilization of the integron (278). In Figure 4 we can find the schematic representation of a typical IS (A), the structure of Tn*2006* implicated in bla_{OXA-23} mobilization (B) and the structure of the Tn*402*-like transposon harboring the bla_{IMP-5} (C).

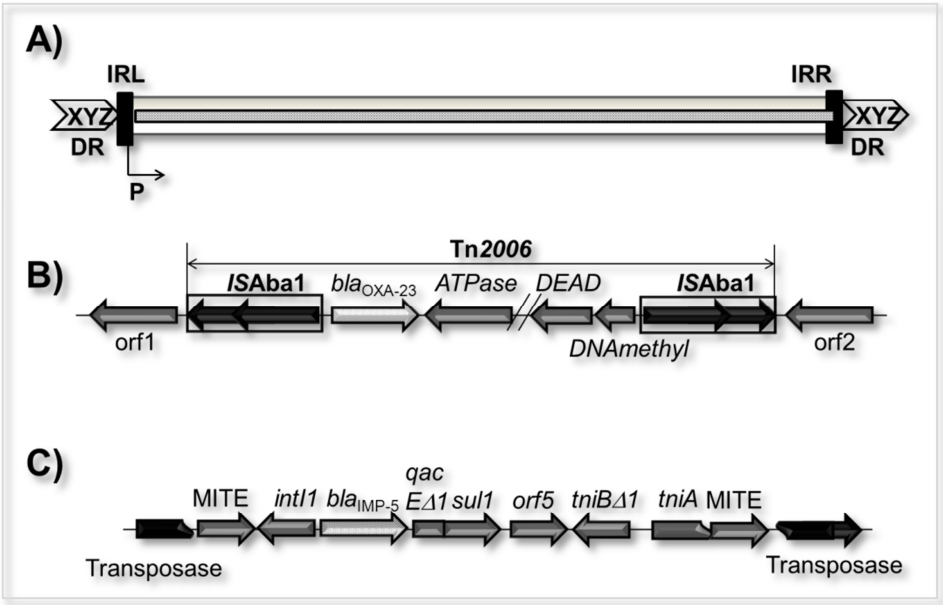

Figure 4. Schematic representation of insertion sequences (IS) and transposons**. A)** Typical IS. IRL, left inverted repeat; IRR, right inverted repeat; transposase is represented by the textured rectangle, extending within IRR and constituting the only ORF of the system; XYZ represent the short direct repeats (DR) sequences generated in the target DNA; P, transposase promotor, partialy located within IRL. (Adapted from Mahillon, 1998) **B)** Tn*2006* structure. The orf 1 and orf2 genes of unknown function are indicated; ATPase, gene encoding the putative AAA ATPase; DEAD, gene encoding the putative DEAD (Asp-Glu-Ala-Asp) helicase; DNAmethyl, DNA methylase. **C)** Schematic

representation of the MITEs structures flanking regions the integron In*76*. [Adapted from (159, 177, 278)]

1.2.1. 2 Integrons

While IS are common vehicles for acquisition of CHDLs genes, MBLs genes such as *bla*$_{IMP}$, *bla*$_{VIM}$ or *bla*$_{SIM}$, but not *bla*$_{NDM}$, are usually embedded in integron structures (223).

Integrons are genetic elements responsible for the acquisition and rearrangement of open reading frames (ORFs), embedded in gene cassette units, guaranteeing genes correct expression. Integrons are not mobile by themselves (but gene cassettes can readily be exchanged to other integrons), but they can spread to different chromosomal locations, plasmids or even other microorganisms by IS-mediated transposition or homologous recombination (165). Acquisition of genetic determinants is usually linked to the so called "mobile integrons" due to their association with mobile DNA elements (transposons), which in turn can be carried by plasmids (32). According to the integrases sequences were identified five different classes of mobile integrons, with class 1 integrons being the most widespread and clinically relevant. Integrons are organized in three regions: the 5′ conserved region, the 3′conserved region, and a variable region. The 5′ region consists of the integrase gene (*intI*), its adjacent recombination site (*attI*), and a promoter, constituting the integron functional platform. The 3′ conserved region often consists of a partially deleted *qac* gene (*qacEΔ1*) fused to a *sul* gene conferring resistance to quartenary ammonium compound and sulfonamide, respectively (270). Figure 5 gives a schematic representation of class1 integrons.

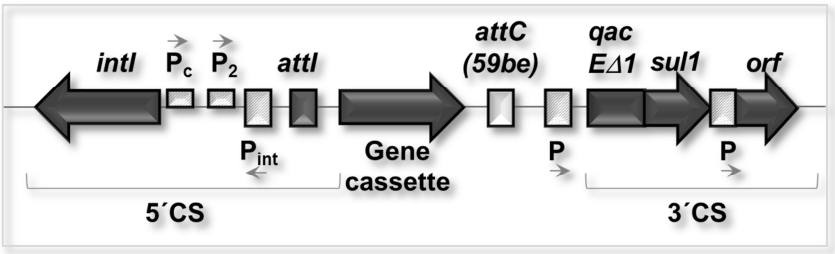

Figure 5. Schematic representation of a class 1 integron. *intI,* integrase gene; Pc cassette promoter; P2, second promoter; Pint, promoter for the integrase; *attI*, integration site; *attC*, sequence in the gene cassette recognized by the integrase; *qacEΔ1*, partially deleted gene that encodes resistance to a quartenary ammonium compound; *sulI*, gene for sulfonamide resistance; orf, open reading frame. [Adapted from (22)]

1.2.1.3 Genomic Islands

Genomic islands constitute clusters of genes involved in horizontal gene transfer (HGT) being generally associated with microbial adaptations of medical or environmental interest (70, 106). They are usually identified due to differences in G+C content from the rest of the genome and by the presence of direct repeats at their ends, the association of pathogenicity islands with transfer RNA genes, the presence of integrase determinants and other mobility loci (106).

The genome sequencing of several *A. baumannii* strains, particularly those belonging to the main epidemic lineages, revealed a high genomic plasticity and the presence of hot-spots for acquisition of genomic islands that might contain resistance markers - resistance islands (131). The best studied of these RIs is AbaR1, described in *A. baumannii* AYE strain and constituting the largest described to date (93). It is an 86 kb region with a G+C content of 52.8% while the G+C typical of *A. baumannii* chromosome is 38.8%. AbaR1 is constituted by 88 predicted ORFs, much of them probably acquired from other species belonging to *Pseudomonas*, *Salmonella* and *Escherichia* genera. AYE strain harbored 52 antibiotic resistance genes with 45 being located in AbaRI. Additionally, this region also includes genetic determinants for resistance to arsenic, mercury and quaternary ammonium compounds (93, 219). Since then several other resistance islands were described among *A. baumannii* isolates being noticed that different clonal lineages seem to display differences in resistance gene content and organization of AbaRs, although most of them are preferentially incorporated into a specific location within the *comM* gene (193, 211). The bla_{OXA-23} is one of the resistance genes found associated with an antibiotic resistance island, Tn*6167*, in an ST92 isolate from Australia (193)

Figure 6 represents the common backbones described for European clone I (ECI) and ECII AbaRs.

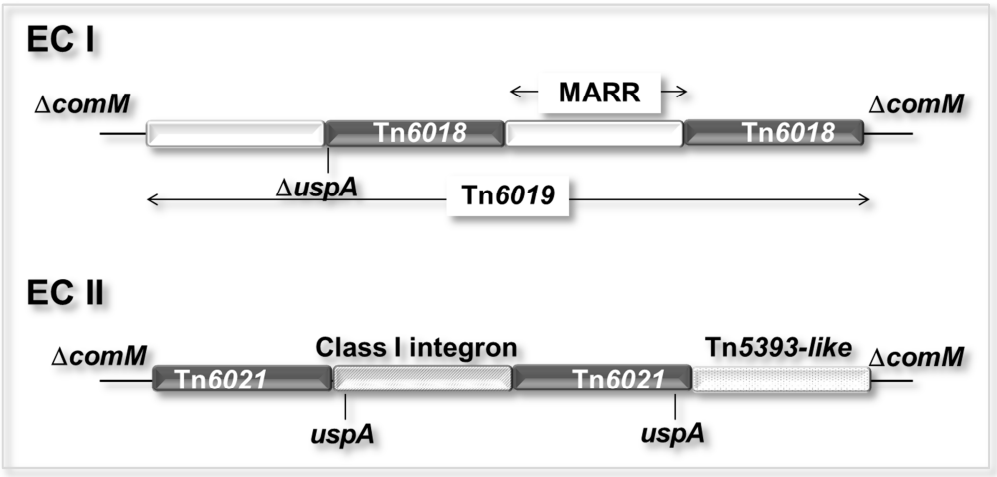

Figure 6. AbaR-type transposons backbones. EC I share a 16.3-kb backbone transposon, Tn*6019*, constituted by two ORFs and the universal stress protein gene (*uspA*) disdisrupted by a second cadmium and zinc resistance transposon (Tn*6018*) which is flanking a multiple antibiotic resistance region (MARR). However is observed higher diversity among AbaRs from EC II isolates. [Adapted from (223)]

1.2.1.4 Plasmids

Plasmids are extra-chromosomal pieces of DNA, which are capable of replicating independently of the genome (241). They play an important role in bacterial adaptation being involved in many accessory functions and constituting the so called “accessory genome”. In general, a plasmid is constituted by one or more genes implicated in replicative functions, and genes coding for different accessory metabolic processes. Plasmid architecture is more flexible than the chromosomal due to the presence of transposable elements giving origin to recombinatory events (92). Most of the plasmids confer positively selectable phenotypes (33) since they are directly implicated in the acquisition of resistance to many antibiotics (241). Additionally, plasmids may cross many species and genus barriers, contributing for a successful dissemination (33, 241).

The development of a classification scheme based on replicons identification revealed that *Acinetobacter* plasmid replicons differ from all those described previously for other prokaryotic species, being identified 27 replicases grouped into 19 homology groups (18).This scheme contributed to better understand the diffusion of plasmids conferring antimicrobial resistance among *Acinetobacter* spp., especially those involved in CHDLs dissemination (18, 250) CHDLs genes were found to be particularly associated with rep*Aci6* and rep*Aci1* (bla_{OXA-23}), rep*Aci6*, rep*Aci1*, rep*Aci3*, rep*Aci4*, and rep*AciX* (bla_{OXA-58}) and rep*Aci6*, rep*Aci2*, and p2ABSDF0001 (bla_{OXA-40}) (250). Curiously, plasmid encoded

bla$_{OXA-40}$ gene was always found to be flanked by conserved inverted repeats homologous to binding sites acting as targets for the XerC and XerD recombinases (57, 169), probably constituting a novel mechanism of mobilization (169).

Thus, *Acinetobacter* spp. successful evolution greatly results from the genomic plasticity provided by the combination of variety of genetic elements.

1.2.2 Horizontal Gene Transfer

Horizontal gene transfer (HGT), in opposition to the transmission of traits from parents to offspring, known as vertical inheritance, corresponds to the transfer of genetic material to the surrounding bacteria, constituting an important source of bacterial adaptation and evolution (81, 251). This event is particularly important in the resistance evolution to antibacterial agents, constituting probably one of the most important means by which the spread of antimicrobial resistance in a hospital environment occurs.

Transformation is considered a major horizontal gene transfer mechanism contributing to genetic adaptation and evolution of prokaryotes (65). *Acinetobacter* spp. belongs to a group of naturally transformable organisms by not discriminating between their own and foreign DNA (78).Conjugation, on the other hand, does not seem to be the most frequent mechanism of acquisition of foreign DNA among this genus, since the most frequent plasmids associated with resistance genes are non-self-transferable (250). The lack of mobilization and transfer functions which is in accordance with the observation that most intrinsic *Acinetobacter* plasmids seem to be non-self-transferable in the laboratory, might be overcome by the almost ubiquitous presence of plasmids belonging to GR6 (*rep*Aci6) that could confer an intrinsic system for plasmid mobilization (18, 250). In addition, was recently demonstrated *in vitro* a new mechanism of carbapenem resistance genes dissemination in *A. baumannii*, via delivery of plasmids harboring *bla*$_{OXA-24}$ gene included in outer membrane vesicles (OMVs) to the surrounding bacteria (227).

1.3 Pathogenesis and virulence factors of *A. baumannii*

There are several factors responsible by the high prevalence of *A. baumannii* strains in the hospital in epidemic and endemic situations but the precise mechanisms that contribute for the establishment and progression of infection are only now being defined.

The most studied mechanism of *A. baumannii* pathogenicity is the biofilm formation, an important requirement for chronic colonization of human tissues and persistence in implanted medical devices (149). Other pathogenic determinants that have been reported in *Acinetobacter* include the production of siderophores such as acinetobactin, with the

ferric-siderophore complex being transported inside bacterial cells with the aid of membrane receptors (TonB-dependent receptors) or hydrolytic enzymes such as phospholipase C and phospholipase D (7, 76, 123). OmpA has also an important role in the pathogenicity as binds to eukaryotic cells, translocates to the nucleus and induces apoptosis (45). Moreover, participates in the biogenesis of *A. baumannii* outer membrane vesicles (OMVs) that interacted with lipid rafts in the plasma membranes and deliver virulence factors, such as tissue-degrading enzymes (124, 175). In addition, *Acinetobacter* growth and resistance to salt stress seems to be enhanced by low concentrations of ethanol which also stimulates the expression of genes encoding proteins involved in siderophore synthesis and siderophore receptors (31).

1.3.1 Biofilm formation

Biofilms are highly structured communities of bacteria attached to a surface and represent the prevalent microbial mode of existence in nature, being suggested that more than 90% of bacteria exist within biofilms (54). Biofilms are associated with multiple drug resistance, being the microorganisms contained in these structures more resistant to desiccation, environmental stress (nutritional or oxidative stress), UV light exposure and phagocytosis. (236) The ability of *A. baumannii* to form biofilms is multifactorial including nutrient availability (eg. iron limitation), bacterial appendages (pili), bacterial surface components (outer membrane proteins, adhesins) quorum sensing and macromolecular secretions (polysaccharides, nucleic acids). The response to a variety of environmental signals and sensing molecules results in differential expression of biofilm associated genes, depending also on the type of surface implicated (97). Table 4 summarizes the main steps involved in biofilm formation.

Table 4. Description of the main mechanisms involved in the development of mature biofilm structures in *A. baumannii*

Stage	Description	Genes/Proteins	References
1 - Adherence	Adhesion to both biotic and abiotic surfaces assisted by type I pili.	CsuA/BABCDE chaperone-usher complex (expression regulated by *bfmR* and *bfmS* genes); Outer membrane proteins (Omp38 and OmpA)	(45, 46, 246, 247)
2 - Irreversible attachment	Cell-to-cell contact: increased production of (c-di-GMP)[a] results in cell-to-cell contact, quorum sensing and reduction in surface associated swarming behavior.	Biofilm-associated protein (Bap)	(149)
3 - Microcolonies	Structured sessile microbial community embedded in an extracellular polymeric substance (EPS) composed of PNAG[b]; eDNA[c], lipids, and proteins. This highly hydrated layer might be responsible for resistance to desiccation.	*pgaABCD* locus	(44)
4 – Cell dispersal	Cell dispersal occurs for daughter cells and when nutrients become limited, being advantageous for bacteria to dissemble and revert to initial planktonic existence. Detachment from surfaces occurs via twitching motility, mediated by type-IV pili.	*pilA, pilB, pilC, pilF, pilM, pilN, pilO, pilP, pilQ, pilR, pilS, pilT, pilU, pilZ, pilW*	(30, 223)

[a]intracellular messenger signalling molecule bis-(3´-5´)-cyclic di-GMP; [b] poly-β- (1-6)-*N*-acetylglucosamine; [c] extracellular DNA

1.4 Population structure of carbapenem-resistant *A. baumannii*

Several typing methods with different discriminatory power have been applied to gain insights into *A. baumannii* population dynamics: Pulsed-Field Gel Electrophoresis (PFGE) – more suitable for short-term outbreak investigations–, amplified fragment length polymorphism (AFLP) analysis, other PCR fingerprinting methods and multiple-locus variable tandem repeat number analysis (MVLA) (9). The development of multilocus sequence typing schemes allowed the generation of objective and comparable data that might be exchanged between laboratories and the establishment of important databases. Presently, there are three MLST schemes in use. The first MLST scheme was developed by Bartual *et al* in 2005 (9), and is hosted in the Oxford database (http://pubmlst.org/abaumannii/). The second MLST scheme, which shares three loci with the original scheme, was developed by Nemec *et al*. at the Pasteur Institute

(http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html) (188). The third one includes PCR coupled with electrospray ionization mass spectrometry (PCR/ESI-MS) (80).

Dissemination of carbapenem-resistant *A. baumannii* seems to be due to the spread of highly similar strains, usually harboring acquired CHDLs. Additionally, inter-hospital transfer of colonized patients might also account for *A. baumannii* outbreaks emphasizing the importance of infection control programs to prevent this dissemination.

In Europe, hospital outbreaks of *A. baumannii* infections have been investigated since the early 1980s (264) Using the AFLP method were identified the European clones (EU) I, II and III that constituted at that time the major genotypes circulating in European hospitals and associated with carbapenem resistance (67, 257) The development of a PCR-based sequence group typing method focused on the allelic variation within three genes likely to be under selective pressure, *ompA*, *csuE* and $bla_{\text{OXA-51-like}}$, created an easier method for the definition of these lineages previously delineated by AFLP (252). More recently, the use of repetitive-sequence-based PCR (rep-PCR) typing with the DiversiLab system has expanded the structure of carbapenem-resistant *A. baumannii* population from three to eight clonal lineages disseminated worldwide (WW1 to -8) (Higgins, 2010). In the particular case of Portugal a $bla_{\text{OXA-40}}$ carbapenem-resistant clone endemic in at least three tertiary-care hospitals (61, 215), formed a sub-cluster of EUII, emphasizing the role of this lineage in carbapenem-resistance dissemination (59).

The use of the MLST scheme developed by Nemec *et al*. at the Pasteur Institute led to the identification of three main clonal complexes (CC1-CC3), each one corresponding to European clones I to III, and ST15, and all associated with the majority of multidrug resistant *A. baumannii* isolates (66).

The MLST Bartual scheme allowed the recognition of the worldwide disseminated clonal complex (CC) 92 which comprises today the majority of carbapenem-resistant *A. baumannii* isolates (9, 96, 110, 115, 179, 202). Among the more than fifty STs included in this CC, ST92 is the most disseminated (Figure 7) being frequently associated with OXA-23 producing *A. baumannii*. In a survey among hospitals from sixteen cities in China was observed the dominance of ST92 among the carbapenem-resistant isolates, with $bla_{\text{OXA-23}}$ being the only CHDL identified in those isolates. Curiously, in that same study, ST92 also achieved a dominant position among the carbapenem-susceptible isolates, raising the possibility that ST92 presents additional features, besides antibiotic resistance, which allow its survival under selective pressures (96). Additionally, in South Korea, ST92-OXA-

23 producing *A. baumannii* clinical isolates were also associated with pan-drug resistance phenotype (203). In Australia, in a study conducted among isolates obtained during eight years was also observed the dominance of ST92 associated with the carriage of bla_{OXA-23} (231). Curiously, in order to avoid a bias, the inclusion of sporadic and susceptible strains in this type of epidemiological studies revealed that the majority of clinical isolates also belong to ST92, reinforcing that its success might be due to an effective adaptation to the hospital environment, as well as a great ability to further acquire antibiotic resistance (231). In a recent survey (2008-2009) from USA hospitals was observed that sequence types (STs) belonging clustering in CC92 and the pan-European clonal lineage II (EUII) were also predominant. The most common were ST122 and ST208, but not ST92, suggesting that carbapenem-resistant *A. baumannii* isolates found in USA hospitals constitute part of the global epidemic driven by CC92, probably sharing the same ancestral, but evolving independently from the European isolates (1).

Several studies confirm the correspondence between CC2 and CC92, with some pointing for the better discrimination obtained with the MLST scheme developed by Bartual et al, 2005 (1, 66, 179, 190). This higher discriminatory power seems to be conferred by the use of *gyrB* and *gpi* loci included in this scheme. However, a report suggesting that horizontal gene transfer has occurred at the *gyrB* and *gpi* loci with some frequency opened the discussion about their utility in phylogeny studies (110).

Figure 7. Worldwide dissemination of *A. baumannii* belonging to ST 92.

1.5 Future perspectives in the control and prevention of *A. baumannii* infections

The extensive antibiotic resistance presented by *A. baumannii,* which has surpassed human capacity to create new antimicrobials, is leading us toward a concerning era of hospital-acquired infections, highlighting the urgent need for novel therapeutic approaches. We will refer just a few strategies that are now being developed, some of them with good efficacy perspectives.

For instance, a renewed interest in phage therapy has led to the characterization of the virulent AB1 bacteriophage which has been shown to be effective against *A. baumannii* (279). Another possible therapeutic option might include the development of vaccines. Several molecules are candidate to be used has antigen: i) inactivated whole cell vaccine seems to elicit a robust antibody response (168); ii) a vaccine consisting on multiple surface antigens from *A. baumannii* outer membrane elicited humoral and cellular responses reducing post infection bacterial loads, and post infection proinflammatory cytokine levels in serum (169); iii) immunization with outer membrane vesicles was also able to elicit protective immunity, particularly in a short period of time (168); and iv) recombinant OmpA using aluminium hydroxide as adjuvant also induced high titers of protective anti-OmpA antibodies (157). However, the use of vaccine against *A. baumannii* raises the question of which population should be immunized. It is proposed that persons presenting risk factors for the development of *A. baumannii* infection, such has long-term hospitalization in intensive care units, mechanical ventilation, and previous antibiotherapy, should be considered as potential targets for vaccination. Other therapeutic options might include the use of radioimmunotherapy, photodynamic or nanoparticle technology (172, 173).

Not all of these approaches will be available in the near future. Prevention and infection control, assisted by good surveillance programs, with an accurately and early recognition of antimicrobial resistance mechanisms, will be the best allied to combat *Acinetobacter* spp. infections.

Chapter 2 OBJECTIVES

"Savoir s'étonner à propos est le premier pas fait sur la route de la découverte."
Louis Pasteur
(1822-1895)

CHAPTER 2 - OBJECTIVES

2.1 Objectives of the study

In the last decades, *Acinetobacter* species have emerged as important and threatening pathogens, representing a significant and difficult challenge for healthcare institutions all over the world. *Acinetobacter baumannii,* the most prevalent *Acinetobacter* species within the clinical setting, shows a prodigious ability to become resistant to several antimicrobial agents and to persist in the hospital environment. With the boost of multidrug resistant strains, carbapenems emerged as last therapeutic options for serious *A. baumannii*-causing infections. Unfortunately, their usefulness rapidly became limited as an increasing number of carbapenem-resistant strains sprouted worldwide.

The emergence and spread of carbapenem-resistant *A. baumannii* within the hospital setting derives mainly from clonal expansion and horizontal gene transfer. These factors together with adhesion and biofilm formation capabilities strongly contributed to the successfully adaptation and evolution of this species. Moreover, the possibility of interspecies transfer of resistance traits could account for carbapenem resistance in *Acinetobacter* species other than *A. baumannii*, which might constitute important source/reservoir of relevant resistance genes.

The **goal** of this thesis was to gain insights on the population diversity of carbapenem-resistant *A. baumannii* clinical isolates*,* the influence of mobile genetic elements in the intra- and inter-species exchange of carbapenem resistance mechanisms, and the role of adhesion and biofilm production in the dissemination and persistence of particular *A. baumannii* lineages. The recognition of the main carbapenem-resistant *A. baumannii* lineages responsible for nosocomial infections as well as the complete understanding of the factors that are driving their dissemination and persistence is essential to create and promote effective strategies to control these organisms within the hospital setting.

With this purpose in mind, the dynamics of *A. baumannii* populations existing in several Portuguese hospitals (1995-2008) was characterized and the role of particular lineages in the dissemination of carbapenem resistance in hospitals from Brazil (2006-2007) and Czech Republic (2008) was determined. Additionally, the role of mobile genetic elements in carbapenem resistance dissemination, not only among *A. baumannii* isolates, but also among different *Acinetobacter* species was also clarified.

This study focuses on the following specific aims:

1. To characterize the population structure of carbapenem-resistant *Acinetobacter baumannii* clinical isolates collected in the hospital setting in Portugal (1995-2008), Brazil (2006-2007) and Czech Republic (2008).
2. To characterize the carbapenem resistance mechanisms and the mobile genetic elements responsible for the spread of carbapenem resistance determinants among *Acinetobacter* species.
3. To explore the mechanisms that might drive the antimicrobial resistance evolution in *A. baumannii*.
4. To explore the role of environmental *Acinetobacter* species in the acquisition and dissemination of carbapenem resistance mechanisms.
5. To characterize the adhesion ability and biofilm formation displayed by the main *A. baumannii* lineages disseminated in Portuguese hospitals.
6. To integrate the results emerging from the objectives 1, 2, 3, 4 and 5 for a better understanding of the role of specific lineages, particular mobile genetic elements and virulence factors in the dynamics of the emergence phenomenon of carbapenem resistance in *Acinetobacter* spp.

2.2 Organization of the thesis

The results gathered during this study were partially published (4 publications in peer review journals, 1 under editorial revision and 2 manuscripts in preparation) and were organized according to the following structure:

I. *Population structure analysis of carbapenem-resistant Acinetobacter baumannii clinical isolates*

This chapter includes results obtained from the characterization process of a large collection of *A. baumannii* isolates collected in the hospital setting in Portugal, Brazil and Czech Republic (1995-2008), as well as the description of *in vivo* evolution of a multidrug-resistant *A. baumannii* isolate towards resistance to all available antibiotics (extreme drug resistance).

- Grosso F, Quinteira S, Peixe L. 2011. Understanding the dynamics of imipenem-resistant *Acinetobacter baumannii* lineages within Portugal. *Clinical Microbiology and Infection*. 17(8):1275-1279.

- Grosso F, Carvalho KR, Quinteira S, Ramos A, Carvalho-Assef AP, Asensi MD, Peixe L. 2011. OXA-23-producing *Acinetobacter baumannii*: a new hotspot of diversity in Rio de Janeiro? *J Antimicrob Chemother.* 66(1):62-5.

- Grosso F, Quinteira S, Pinheiro M, Bergerová T, Hrabák J, Peixe L. Clonal outbreak of Multidrug-Resistant ST92 *Acinetobacter baumannii* in the University Hospital of Pilsen, Czech Republic. (*in preparation*)

- Grosso F, Quinteira S, Peixe L. 2010. Emergence of an extreme-drug-resistant (XDR) *Acinetobacter baumannii* carrying $bla_{\text{OXA-23}}$ in a patient with acute necrohaemorrhagic pancreatitis. *Journal of Hospital Infection*. 75(1):82-83.

II. Contribution *of mobile genetic elements for the dissemination of carbapenem resistance among Acinetobacter spp.*

The characterization of $bla_{\text{OXA-24/40}}$-carrying plasmids and $bla_{\text{IMP-5}}$ genetic environments, exploring the role of horizontal gene transfer in the dissemination of carbapenem resistance, was conducted and the collected data is presented and discussed within this chapter.

- Grosso F, Quinteira S, Poirel L, Novais A, Peixe L. 2012. The role of common *bla*$_{OXA-24/40}$-carrying platforms and plasmids in the spread of OXA-24/40 among *Acinetobacter* spp. clinical isolates. *Antimicrobial Agents and Chemotherapy*. 56(7):3969-3972.
- Grosso F, Ramos H, Quinteira S, Peixe L. IMP-5-producing multidrug-resistant *Acinetobacter lwoffii* from a newborn incubator. (*submitted*)

III. *Contribution of adhesion and biofilm production for the persistence of carbapenem-resistant A. baumannii lineages*

In this study the biofilm formation ability of spread carbapenem resistant *A. baumannii* lineages was investigated. The possible role of *bla*$_{OXA-24/40}$-carrying plasmids as a genetic platform for the anchorage of biofilm formation capabilities, as well as its contribution for the endemicity of some lineages in Portugal, is also discussed.

- Vuotto C, Grosso F, Costa ML, Ferreira H, Quinteira S, Donelli G, Peixe L. Contribution of adhesion and biofilm production in the persistence of carbapenem-resistant *A. baumannii lineages*: exploring the role of *bla*$_{OXA-24/40}$-carrying plasmids. (*in preparation*)

Chapter 3 RESULTS

"In the fields of observation chance favors only the prepared mind."
Louis Pasteur
(1822-1895)

I. *Population structure analysis of carbapenem-resistant Acinetobacter baumannii clinical isolates*

- Grosso F, Quinteira S, Peixe L. 2011. Understanding the dynamics of imipenem-resistant *Acinetobacter baumannii* lineages within Portugal. *Clinical Microbiology and Infection*. 17(8):1275-1279.

- Grosso F, Carvalho KR, Quinteira S, Ramos A, Carvalho-Assef AP, Asensi MD, Peixe L. 2011. OXA-23-producing *Acinetobacter baumannii*: a new hotspot of diversity in Rio de Janeiro? *J Antimicrob Chemother.* 66(1):62-5.

- Grosso F, Quinteira S, Pinheiro M, Bergerová T, Hrabák J, Peixe L. Clonal outbreak of Multidrug-Resistant ST92 *Acinetobacter baumannii* in the University Hospital of Pilsen, Czech Republic. (*in preparation*)

- Grosso F, Quinteira S, Peixe L. 2010. Emergence of an extreme-drug-resistant (XDR) *Acinetobacter baumannii* carrying $bla_{\text{OXA-23}}$ in a patient with acute necrohaemorrhagic pancreatitis. *Journal of Hospital Infection*. 75(1):82-83.

Understanding the dynamics of imipenem-resistant *Acinetobacter baumannii* lineages within Portugal.

Filipa Grosso[1], Sandra Quinteira[1,2] and L. Peixe[1]

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

2) Centro de Investigação em Tecnologias da Saúde (CITS)/IPSN (CESPU), VN Famalicão, Portugal

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Understanding the dynamics of imipenem-resistant *Acinetobacter baumannii* lineages within Portugal

F. Grosso[1], S. Quinteira[1,2] and L. Peixe[1]

1) *REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto and 2) Centro de Investigação em Tecnologias da Saúde (CITS)/IPSN (CESPU), VN Famalicão, Portugal*

Abstract

A recent collection of 213 imipenem-resistant *Acinetobacter baumannii* (IRAB) clinical isolates was characterized for the presence of acquired carbapenem-hydrolysing class D β-lactamases (CHDLs) and clonality. A population structure analysis of IRAB was also conducted, with five molecular typing methods. Three main clusters, each one associated with a specific CHDL, were observed with multilocus sequence typing. Overall, our results suggest a switch in the dominant clone, with sequence type (ST) 92, carrying bla_{OXA-23} (63.4%), replacing the closely related ST98, carrying $bla_{OXA-24/40}$ (22%). In addition, ST103, an independent lineage, was associated with bla_{OXA-58}-carrying isolates (14.6%).

Keywords: bla_{OXA-51}-like, carbapenemase, multilocus sequence typing, pulsed-field gel electrophoresis type, sequence groups

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Corresponding author: L. Peixe, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164 4050-047 Porto, Portugal
E-mail: lpeixe@ff.up.pt

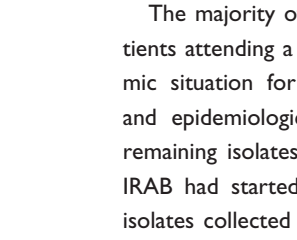
During the last decade, an ongoing rise in *Acinetobacter baumannii* carbapenem resistance has been observed worldwide, with recent reports of resistance to all available antimicrobials [1,2]. The production of acquired carbapenem-hydrolysing class D β-lactamases (CHDLs) belonging to the OXA-23, OXA-24/40 and OXA-58 groups is the main reported carbapenem resistance mechanism [1–7]. Despite the description of two particular isolates producing IMP-5 [8] and, more recently, OXA-23 [2], the imipenem resistance observed in Portuguese *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 [3,6]. Several sequence-based typing methods have been proposed for *A. baumannii* population structure analyses [9–12], with multilocus sequence typing (MLST) being increasingly applied as a standard method [4,5,10,11,13]. Nevertheless, studies evaluating the robustness of each particular method are still scarce.

In this work, we have characterized the antimicrobial susceptibility, acquired CHDLs and clonality of 213 previously undescribed imipenem-resistant *A. baumannii* (IRAB) isolates,

collected from 2004 to 2008, from five hospitals and two clinical analysis laboratories located in the north and central regions of Portugal. Isolates were identified with the Vitek 2 system and 16S rRNA gene sequencing. Susceptibility to β-lactams, colistin and tigecycline was determined according to CLSI guidelines [14]. The bla_{OXA} genes were detected with a multiplex PCR [7] and confirmed by sequencing. The genetic location of acquired CHDL genes was assessed by hybridization of I-*Ceu*I-digested genomic DNA with specific probes for bla_{OXA-23}, $bla_{OXA-24/40}$, bla_{OXA-58} and 16S rRNA [6]. The vicinity of each CHDL gene was established by PCR and sequencing [15–18]. Pulsed-field gel electrophoresis (PFGE) typing was performed with the *Apa*I restriction enzyme [3], and pattern analysis was conducted with InfoQuestTM FP v5.4 (BioRad Laboratories, Hercules, CA, USA). Isolates clustering together with >85% similarity levels [19] were considered to belong to the same PFGE type. In order to better understand the population structure of CHDL-producing IRAB Portugal, we also used four distinct sequence-based typing methods (Table 1). To this end, we selected nine representative isolates (from the 213 IRAB isolates described above), together with four additional isolates from a previously characterized collection [3,6]. This selection process was aimed at simultaneously gathering isolates with different acquired CHDL content and of distinct PFGE types, collected from different hospitals over the course of several years. Identification of PCR-based sequence groups (SGs) and sequence-based typing of bla_{OXA-51}-like genes were performed as described elsewhere [9,12]. MLST analysis was conducted according to two distinct schemes that will be referred to from now on as MLST1 (developed by Bartual *et al.* [10]) and MLST2 (published by Nemec *et al.* [11]). Clonal complexes (CCs) for MLST1 were determined with the eBURST software program (http://eburst.mlst.net/) [20]. It is important to point out the renumbering of alleles, sequence types (STs) and CCs that occurred on the MLST1 database in February 2010. CCs relating to MLST2 were deduced with a minimum spanning tree analysis [13].

The majority of isolates (77.5%) were obtained from inpatients attending a general hospital (hospital A), with an endemic situation for IRAB, continuously observed since 2001 and epidemiologically monitored by our group [3,6]. The remaining isolates were provided by four hospitals in which IRAB had started to spread more recently (Table 1). Two isolates collected from distinct outpatients, with no previous hospitalization history, were obtained from two clinical analysis laboratories. A predominance of bla_{OXA-23} has been observed since 2006, being detected in 135 isolates (63.4%) from four hospitals and from two outpatients; this represents, to our knowledge, the first description of

TABLE 1. Characteristics of imipenem-resistant *Acinetobacter baumannii* clinical isolates and relationship between carbapenem-hydrolysing class D β-lactamase (CHDL) content, pulsed-field gel electrophoresis (PFGE) types, sequence groups (SGs), bla_{OXA-51}-like types and the results of two multilocus sequence typing (MLST) schemes



Isolates (n)[a]	Date	Origin (region)	MIC range (mg/L)[b]				CHDL	PFGE	bla_{OXA-51}-like	Sequence-based typing methods (n = 13)					References
			IPM	MEM	COL	TIG				SG	MLST1[c] ST	MLST1[c] CC	MLST2[d] ST	MLST2[d] CC	
Ac246 (28)	2004	Hospital A (north)	>32	8 to >32	0.5–2	1–4	OXA-58	B	$bla_{OXA-132}$	4	103	–	15	–	This study
HGSA140 (1)	2008	Hospital A (north)	>32	>32	2	4	OXA-58	B	$bla_{OXA-132}$	4	103	–	15	–	This study
Ac156 (2)	2004	Hospital A (north)	32	4	2–4	1–4	OXA-58	C	$bla_{OXA-132}$	4	103	–	15	–	This study
Ac92 (63)	2003	Hospital A (north)	>32	>32	0.5 to >16	1–8	OXA-24/40	A	bla_{OXA-66}	–	98	92	2	2	[6]
HGSA55 (3)	2008	Hospital A (north)	>32	>32	0.5 to >16	4–16	OXA-24/40	A	bla_{OXA-66}	–	98	92	2	2	This study
HGSA56 (92)	2007–2008	Hospital A (north)	>32	>32	0.5 to >16	4–16	OXA-23	A	bla_{OXA-66}	–	92	92	2	2	This study
HPH7 (21)	2006–2007	Hospital C (north)	>32	>32	0.5–2	16–32	OXA-23	A	bla_{OXA-66}	–	92	92	2	2	This study
141FFC (23)	1999–2001	Hospital G (centre)	>32	>32	ND	ND	OXA-24/40	A	bla_{OXA-66}	–	98	92	2	2	[3]
Ac2 (19)	2001	Hospital A (north)	>32	>32	0.5–2	2–4	OXA-24/40	A	bla_{OXA-66}	–	98	92	2	2	[6]
988FFP (1)	1995	Hospital B (north)	>32	>32	ND	ND	OXA-24/40	A	bla_{OXA-66}	–	98	92	2	2	[3]
CHCB10 (5)	2007	Hospital F (centre)	>32	>32	4	16	OXA-24/40	A	bla_{OXA-66}	–	98	92	2	2	This study
HGSA 7 (39)	2006	Hospital A (north)	>32	>32	0.5 to >16	4–16	OXA-24/40	A	bla_{OXA-66}	–	98	92	2	2	This study
HST 1 (14)	2006–2008	Hospital E (centre)	>32	>32	>16	16–64	OXA-23	A	bla_{OXA-66}	–	92	92	2	2	This study, [2]

CC, clonal complex; COL, colistin; IPM, imipenem; MEM, meropenem; ND, not determined; TIG, tigecycline; ST, sequence type.
[a]n, total of isolates collected during the same period sharing similar characteristics; bold refers to isolates characterized in this study.
[b]MICs of imipenem and meropenem were determined by Etest (AB Biodisk, Solna, Sweden), and those of tigecycline and colistin were determined by the agar dilution method [14].
[c]MLST1, MLST scheme developed by Bartual *et al.* [10] (http://pubmlst.org/abaumannii/).
[d]MLST2, MLST scheme developed by Nemec *et al.* [11] (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html).
The similarity percentage of profiles was calculated applying the Dice coefficient. The optimizational and positier tolerance for band analysis were set at 10% and 15% respectively.

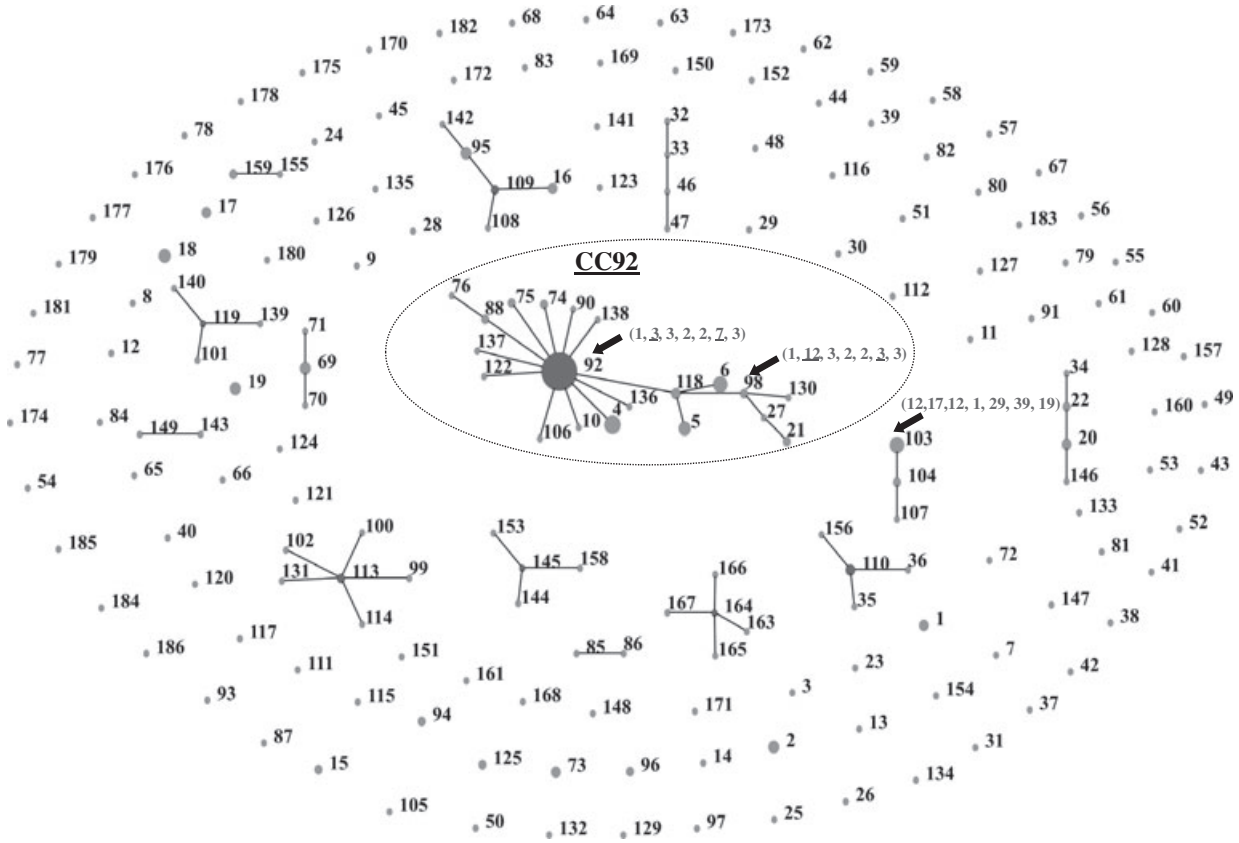


FIG. 1. Clustering of *Acinetobacter baumannii* sequence types (STs) by eBurst, with 185 multilocus sequence typing profiles representing 304 isolates from the database (http://pubmlst.org/abaumannii/, last accessed 30 July 2010). Each ST is represented as a node; the relative size of each node is indicative of its prevalence among the isolates, and lines connect single-locus variants. Clonal complex (CC)92 is represented by a dashed circle. STs and the corresponding allelic profiles for the 13 isolates included in the population structure study are indicated by arrows.

bla_{OXA-23}-producing *A. baumannii* within the community. Overall, besides resistance to all tested β-lactams, including carbapenems, they had higher tigecycline MICs (Table 1), findings that might explain their dominance. In fact, one isolate even showed an extreme drug resistance phenotype [2]. All OXA-23-producing isolates showed the association of bla_{OXA-23} with Tn*2006*, with a chromosomal location. The $bla_{OXA-24/40}$ gene was detected in 47 isolates (22%) from two hospitals. This acquired CHDL gene was encoded in plasmids, flanked by XerC/XerD-like binding sites [18] and, for some isolates, it was also observed on the chromosome (Gross. F, Quinteila S, Peixe L, unpublished data). The bla_{OXA-58} gene was detected in 31 isolates (14.6%) obtained from hospital A. The higher susceptibility generally shown by these isolates, namely to meropenem and tigecycline, might explain their low prevalence. The bla_{OXA-58} gene was flanked by *IS*Aba3-like and *IS*Aba3 elements upstream and downstream, respectively, and was found to be both chromosomal and plasmid-carried. The insertion sequence *IS*Aba1 was observed upstream of bla_{OXA-51}-like in all isolates. Only three PFGE types were identified within this IRAB population: PFGE type A for bla_{OXA-23}-carrying and $bla_{OXA-24/40}$-carrying IRAB, and PFGE types B and C for bla_{OXA-58}-carrying IRAB (Table 1). Identification of SGs showed that both bla_{OXA-23}-carrying and $bla_{OXA-24/40}$-carrying IRAB isolates belonged to SG1 [9], which corresponds to international clone II [13]. These isolates also harboured the same bla_{OXA-51}-like gene, bla_{OXA-66}, and belonged to ST2 (CC2) as revealed by the MLST2 scheme [11], suggesting a close relationship between these two groups of isolates. The bla_{OXA-58}-carrying IRAB isolate belonged to SG4 [19], harboured $bla_{OXA-132}$ as a bla_{OXA-51}-like allele, and belonged to ST15 according to the MLST2 scheme. When the MLST1 scheme [10] was applied, it was possible to observe a distinction between bla_{OXA-23}-carrying and $bla_{OXA-24/40}$-carrying IRAB on the basis of *gpi* and *gyr*B allelic variants. Thus, ST92 (the former ST22) was identified for bla_{OXA-23}-carrying IRAB, and a novel ST, ST98 (formerly designated ST33, and double-locus variant of ST92) was identified for $bla_{OXA-24/40}$-carrying IRAB (Table 1; Fig. 1). These STs were included together in the

worldwide predominant CC92, with 20 recognized STs, ST92 being the most frequently reported and disseminated [4,5] (http://pubmlst.org/abaumannii/). The available data indicate that most of the *bla*-$_{OXA-23}$-carrying IRAB isolates cluster with ST92 [4,5], and are usually associated with multidrug resistance patterns, including reports of extreme drug resistance [1]. On the other hand, ST98 seems to be associated only with *bla*$_{OXA-24/40}$-carrying IRAB from Portugal (http://pubmlst.org/abaumannii/). Moreover, with this MLST1 scheme, *bla*$_{OXA-58}$-carrying IRAB isolates were associated with ST103 (formerly designated ST38), a lineage with no apparent relationship with CC92 (Table 1; Fig. 1).

In summary, our results show the current dominance of the worldwide ST92 carrying *bla*$_{OXA-23}$. It seems interesting to highlight the progressive switching that occurred in our country, as shown by the MLST1 scheme, where ST98 IRAB carrying *bla*$_{OXA-24/40}$ has been gradually replaced by ST92 IRAB carrying *bla*$_{OXA-23}$. This lineage, which exhibits an extensive multidrug resistance profile, is now emerging in hospitals that have never before experienced the occurrence of IRAB. It is well known that the association of CHDL genes with mobile genetic elements favours transference between different lineages. Curiously, we observed stability of a specific acquired CHDL within each lineage. Although both MLST schemes have been applied to differentiate *A. baumannii* populations [4,5,10,11,13], our data point to the higher resolution of the MLST1 scheme, which provided a better association between epidemiological features, acquired CHDL content and temporal distribution of the studied isolates. It is reasonable to suggest that this MLST scheme could emerge as a key epidemiological tool for future studies of the regional and global epidemiology of *A. baumannii*.

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Transparency Declaration

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Multiple ST clonal complexes, with a predominance of ST131, of *Escherichia coli* harbouring $bla_{CTX\text{-}M\text{-}15}$ in a tertiary hospital in Tanzania

S. E. Mshana[1], C. Imirzalioglu[2], T. Hain[2], E. Domann[2], E. F. Lyamuya[3] and T. Chakraborty[2]

1) Weill Bugando University College of Health Sciences, Mwanza, Tanzania, 2) Institute of Medical Microbiology, Giessen, Germany and 3) Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania

Abstract

The molecular epidemiology of 32 non-duplicate, CTX-M-15 extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* strains, isolated from clinical samples, was investigated. Multilocus sequence typing revealed multiple sequence type clonal complexes: ST131 (12), ST405 (4), ST638 (3), ST38 (2), ST827 (2), ST224 (1), ST648 (1), ST46 (1) and two new sequence type clonal complexes (1845 and 1848) in 22 pulsed field gel electrophoresis clusters. The $bla_{CTX\text{-}M\text{-}15}$ gene was located on conjugative IncF plasmids. This is the first report of the worldwide emerging clonal complex ST131 linked to $bla_{CTX\text{-}M\text{-}15}$ in Tanzania and demonstrates the need for constant surveillance in developing countries to prevent the spread of these multiresistant isolates.

Keywords: $bla_{CTX\text{-}M\text{-}15}$, *Escherichia coli*, ST131, Tanzania

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Corresponding author: C. Imirzalioglu, Justus–Liebig University of Giessen, Institute of Medical Microbiology, Frankfurter Strasse 107, D-35392 Giessen, Germany
E-mail: can.imirzalioglu@mikrobio.med.uni-giessen.de

Extended-spectrum beta-lactamases (ESBLs) are the predominant resistance enzymes among enterobacteriaceae and most of the beta-lactamases described to date are plasmid-encoded enzymes [1,2]. CTX-M is a recently described family of ESBLs. These enzymes hydrolyze cefotaxime more effectively than ceftazidime [3] and are also able to hydrolyze cefepime with high efficiency. The $bla_{CTX\text{-}M\text{-}15}$ allele is considered to be the predominant allele worldwide [4,5]. Extensive studies investigating the association of the multilocus sequence typing (MLST) clonal complex ST131 and $bla_{CTX\text{-}M\text{-}15}$ have been reported from Canada, India, Kuwait, France, Switzerland, Portugal, Spain, Korea and Japan; worldwide dissemination of $bla_{CTX\text{-}M\text{-}15}$ seems to be linked to this clonal complex, which is situated in the phylogenetic group B2 [4,5].

Two studies revealed that CTX-M enzymes were present among *Enterobacteriaceae* in a few isolates in Tanzania [6,7]. However, no study has investigated molecular characteristics of *Escherichia coli* ESBL-producing isolates in detail from this region of the world. For the first time we report the existence of the ST131 and multiple other sequence type (ST) clones carrying $bla_{CTX\text{-}M\text{-}15}$ in a single tertiary hospital in Tanzania, East Africa.

A total of 32 consecutive antimicrobial-resistant *Escherichia coli* clinical isolates were recovered from various wards and clinics in a tertiary hospital in Tanzania. Of these, 27 were from inpatients who had been on the ward for more than 72 h when the specimens were collected, whereas five strains were obtained from outpatients. Strains were identified as *E. coli* using in-house biochemical profiles [8]. Susceptibility patterns to ampicillin, amoxicillin/clavulanic acid, sulphamethaxazole/trimethoprim, tetracycline, gentamicin, ciprofloxacin, ceftriaxone, cefotaxime, cefepime and meropenem were determined by disk diffusion [9]; a disk synergy test was performed to detect ESBL production [8]. *Escherichia coli* ATCC 25922 was used as the quality control strain.

PCR amplification of *bla* genes (bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$) was performed using primers and methods described previously [10]. All PCR products were sequenced (LGC genomics GmbH, Berlin, Germany) and the resulting sequences were compared with known sequences using DNASTAR software (DNASTAR Inc, Madison, WI, USA) and the Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, Bethesda, MA, USA).

OXA-23-producing *Acinetobacter baumannii*: a new hotspot of diversity in Rio de Janeiro?

Filipa Grosso[1], Karyne Rangel Carvalho[2], Sandra Quinteira[1,3], Ana Ramos[1], Ana Paula D'Alincourt Carvalho-Assef[2], Marise Dutra Asensi[2] and Luísa Peixe[1]

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

[2]Laboratório de Pesquisa em Infecção Hospitalar, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil;

[3]Centro de Investigação em Tecnologias da Saúde (CITS)/IPSN (CESPU), Vila Nova de Famalicão, Portugal

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OXA-23-producing *Acinetobacter baumannii*: a new hotspot of diversity in Rio de Janeiro?

Filipa Grosso[1], Karyne Rangel Carvalho[2], Sandra Quinteira[1,3], Ana Ramos[1], Ana Paula D'Alincourt Carvalho-Assef[2], Marise Dutra Asensi[2] and Luísa Peixe[1]*

[1]*REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal;* [2]*Laboratório de Pesquisa em Infecção Hospitalar, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil;* [3]*Centro de Investigação em Tecnologias da Saúde (CITS)/IPSN (CESPU), VN Famalicão, Portugal*

*Corresponding author. Tel: +351-222078946; Fax: +351-222003977; E-mail: lpeixe@ff.up.pt

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Objectives: This study focused on the population structure of OXA-23-producing *Acinetobacter baumannii* clinical isolates from Rio de Janeiro, Brazil.

Methods: The analysis included several genomic typing methods, including PFGE, two multilocus sequence typing (MLST) schemes, sequence group (SG) determination and $bla_{\text{OXA-51-like}}$ sequencing. The genomic context of the $bla_{\text{OXA-23}}$ gene was also evaluated using I-CeuI hybridizations and PCR assays.

Results: Congruent clustering was obtained revealing four lineages. In accordance, four new sequence types (STs) (ST131, ST132, ST133 and ST134) were obtained with the MLST-OD scheme (associated with the Oxford Database) and four (ST79, ST15 and two new allelic profiles) with the MLST-IP scheme (developed by the Institute Pasteur). Four SGs (SG1, SG4 and two new profiles) were identified, allowing the association of 70% of the isolates with European clone II. $bla_{\text{OXA-51-like}}$ sequencing revealed the presence of $bla_{\text{OXA-66}}$, $bla_{\text{OXA-69}}$, $bla_{\text{OXA-95}}$ and $bla_{\text{OXA-132}}$.

Conclusions: Identification of new STs together with new SG profiles are findings suggestive of a local diversity hotspot that is worth exploring.

Keywords: $bla_{\text{OXA-23}}$, PFGE, MLST, sequence groups, $bla_{\text{OXA-51-like}}$

Introduction

In recent years, Brazil has faced the emergence of multidrug-resistant *Acinetobacter baumannii* with some reports implicating the production of OXA-23 as the main carbapenem resistance mechanism.[1–3]

Although several methodologies have been proposed for assessing the genomic diversity of carbapenem-resistant *A. baumannii* (CRAB) isolates, the recently developed multilocus sequence typing (MLST) seems to represent a reliable tool for providing consistent data on its epidemiology, especially due to the possibility of results being compared between different laboratories by easy access to international databases.[4,5] Recent MLST studies on the clonal diversity of $bla_{\text{OXA-23}}$-carrying isolates revealed their association mainly with sequence type (ST) 92 (formerly designated ST22) and with European clone II (EU-II).[6,7] A single description of an OXA-23-producing isolate from Brazil by MLST,[7] and studies reporting genomic diversity among OXA-23-carrying CRAB determined by PFGE,[1,3] constitute the only available information regarding the Brazilian CRAB population structure. Moreover, the relationship of most of these isolates to the major CRAB lineages disseminated worldwide,[8] namely EU-I, EU-II and EU-III, is still unknown.

The present study reports the characterization of the population structure of a previously published collection of OXA-23-producing *A. baumannii* isolates disseminated throughout Rio de Janeiro, Brazil.[1]

Material and methods

In this study, 96 OXA-23-producing *A. baumannii* clinical isolates collected between January 2006 and September 2007, from eight hospitals in Rio de Janeiro, Brazil, were included.[1] PFGE typing was performed as previously described,[9] using ApaI restriction enzyme, and the patterns obtained were analysed by InfoQuestTM FP version 5.4 software (Bio-Rad Laboratories) with Dice coefficient analysis of peak positions executed. The unweighted-pair group method using average linkages was applied and the bandwidth tolerance was set at 1.5%. Isolates clustering together with >85% level of similarity were considered to belong to the same PFGE type.[10] The PFGE types obtained were additionally

compared with representative isolates that belong to ST92 (mainly OXA-23 producers), ST98 (OXA-40 producers) and ST103 (OXA-58 producers) from Portugal and the Czech Republic (F. Grosso, S. Quinteira and L. Peixe, unpublished results).

One isolate representing each of these PFGE types was selected and submitted to sequence-based typing methods including PCR-based sequence group (SG) identification,[11] $bla_{\text{OXA-51-like}}$ sequencing[12] and two MLST schemes.[4,5] Identification of PCR-based SGs was conducted in order to establish the association with the European *A. baumannii* clones [EU-I (SG2), EU-II (SG1) and EU-III (SG3)].[8,10,11] Sequence-based typing of $bla_{\text{OXA-51-like}}$ genes was also performed as described previously.[12] MLST analysis was conducted according to two distinct schemes that will be referred to from now on as MLST-OD (associated with the Oxford Database) and MLST-IP (developed by the Institute Pasteur).[4,5] Nucleotide sequences were submitted to the *A. baumannii* MLST databases (MLST-OD, http://pubmlst.org/abaumannii/; and MLST-IP, http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html) for assignment of allele numbers and STs. Isolates with five or more identical alleles (MLST-OD) were considered part of the same clonal complex (CC), according to the eBURST software program (http://eburst.mlst.net/).[13] CCs based on MLST-IP were deduced with a minimum spanning tree analysis of *A. baumannii* isolates.[8]

The genetic location of the $bla_{\text{OXA-23}}$ gene was identified using I-CeuI digestion, transfer and hybridization with specific probes for $bla_{\text{OXA-23}}$ and 16S rRNA.[14] The association of the $bla_{\text{OXA-23}}$ gene with the composite transposon Tn*2006* was determined using a PCR assay.[14]

Results and discussion

The 96 OXA-23-producing *A. baumannii* were grouped into four PFGE types showing no relationship to representative carbapenem-resistant isolates that belong to ST92, ST98 and ST103 (data not shown). The four clusters arising from PFGE typing were also discerned by sequence-based typing methods. With MLST-OD four new STs were detected: ST131, ST132, ST133 and ST134 (Table 1 and Figure 1). These new allelic profiles were not related to the worldwide-predominant ST92/OXA-23 producers described in several European countries, Korea and China (F. Grosso, S. Quinteira and L. Peixe, unpublished results).[6,7] Moreover, three of the new STs are singletons (Table 1 and Figure 1). ST79, ST15 and two new allelic profiles were detected when MLST-IP was applied (Table 1). The SGs identified were SG1 (the most frequently described among OXA-23-producing isolates),[7] SG4 and two new profiles (Table 1). The $bla_{\text{OXA-51-like}}$ sequencing approach revealed the presence of $bla_{\text{OXA-66}}$ (the most widespread), $bla_{\text{OXA-69}}$, $bla_{\text{OXA-95}}$ and $bla_{\text{OXA-132}}$ (Table 1).

PFGE type A (70% of the OXA-23-producing isolates found within seven hospitals) was assigned to ST131 (MLST-OD), ST79 (MLST-IP) and SG1, and presented the $bla_{\text{OXA-66}}$ gene. ST131 belongs to CC113, which includes STs found in Argentina (Figure 1). Although belonging to SG1 (EU-II), ST79 is not closely related to either CC2 or ST15. These lineages were described recently as widespread clonal clusters that include most of the CRAB isolates.[8] PFGE type B (25% of isolates) was assigned to ST133 (MLST-OD), ST15 (MLST-IP) and SG4, and presented the $bla_{\text{OXA-132}}$ gene, only previously reported in Saudi Arabian[15] and Portuguese isolates (F. Grosso, S. Quinteira and L. Peixe, unpublished results). The identification of ST15 as an emergent international multidrug-resistant clone[8] and recently reported for another Brazilian OXA-23-producing *A. baumannii*[7] is worthy of note.

Table 1. Relationship between PFGE types, STs, $bla_{\text{OXA-51-like}}$ types and SGs obtained for OXA-23-producing *A. baumannii* clinical isolates

Years	No. of hospitals[a]	PFGE type	n[b] (% of isolates)	Sequence-based typing method ($n=4$)[c]					
				MLST-OD[d]		MLST-IP[e]			
				allelic profile (ST)	CC	allelic profile (ST)	CC	$bla_{\text{OXA-51-like}}$ gene	SG (allelic profile)
2006–07	7	A	67 (70)	1-15-60-10-28-56-32 (131)	CC113	26-2-2-2-29-4-5 (79)	singleton	$bla_{\text{OXA-66}}$	1
2006–07	5	B	24 (25)	12-17-72-1-29-67-39 (133)	singleton	6-6-8-2-3-5-4 (15)	ST15	$bla_{\text{OXA-132}}$	4
2007	2	C	4 (4)	1-12-71-2-1-79-30 (132)	singleton	3-2-2-2-2-4-8 (new 1)	singleton	$bla_{\text{OXA-95}}$	new (SG1 $bla_{\text{OXA-66}}$ + SG2 *ompA* and SG2 *csuE*)
2007	1	D	1 (1)	2-12-73-12-1-9-47 (134)	singleton	3-1-6-2-4-1-5 (new 2)	singleton	$bla_{\text{OXA-69}}$	new (SG2 *ompA* + SG2 $bla_{\text{OXA-69}}$)

[a]No. of hospitals, total no. of hospitals where each clone was identified.
[b]n, total no. of isolates.
[c]Sequence-based typing methods were performed for four isolates, representative of the four PFGE types.
[d]MLST-OD, MLST scheme developed by Bartual *et al.*[4]
[e]MLST-IP, MLST scheme developed by Nemec *et al.*[5]

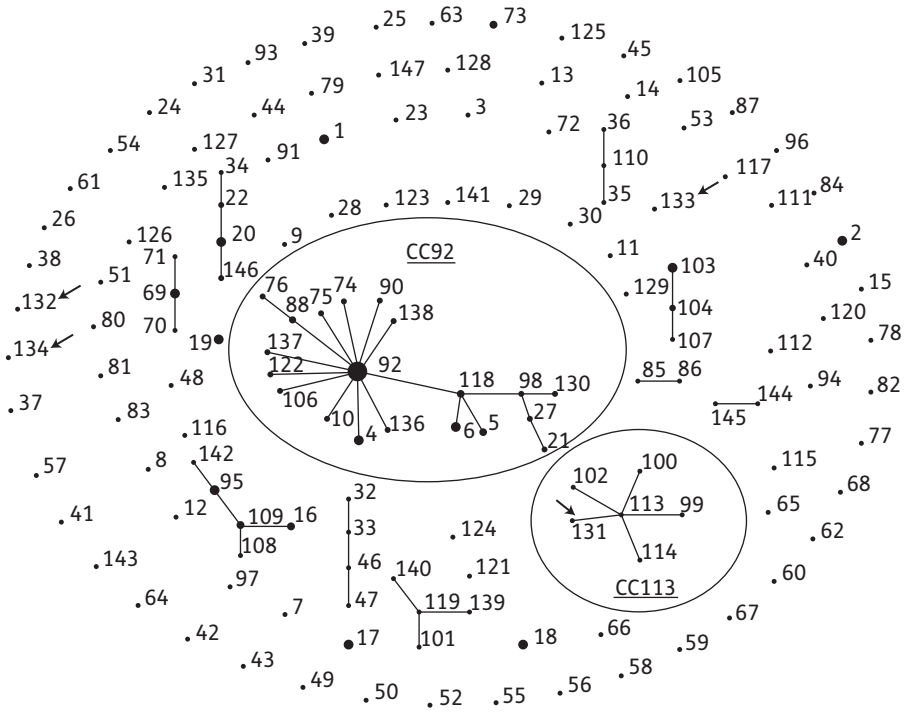

Figure 1. Clustering of *A. baumannii* STs (MLST-OD) by eBurst, with 185 MLST profiles representing 304 isolates from the database (http://pubmlst.org/abaumannii/; 30 June 2010, date last accessed). Each ST is represented as a node (the relative size of each node is indicative of its prevalence among the isolates) and lines connect single-locus variants. CC92 and CC113 are shown in circles. STs determined for isolates from the present study are indicated by arrows.

The representative isolate of PFGE type C (4% of isolates) belonged to ST132 (MLST-OD), revealed new allelic profiles when MLST-IP and SG identification were applied and presented the $bla_{\text{OXA-95}}$ gene. Finally, PFGE type D (1% of the isolates) was assigned to ST134 (MLST-OD), also revealed new allelic profiles with MLST-IP and SG identification, and presented the $bla_{\text{OXA-69}}$ gene (Table 1).

We identified the presence of four unrelated OXA-23-producing *A. baumannii* clonal lineages currently causing infections in Rio de Janeiro hospitals. Interestingly, despite the clonal diversity, the $bla_{\text{OXA-23}}$ gene was consistently found associated with Tn*2006* and was chromosomally encoded in all isolates.

Even considering that the currently used sequence-based typing methods can differ in their intrinsic degree of robustness, a factor that might ultimately impair their discriminatory power, it should be stressed that the observed pattern of results was coherent, independent of the selected methodology, always pinpointing the existence of congruent clusters of isolates characterized by very distinctive features. As the application of these methods becomes more widely established, a process of 'natural selection' will surely narrow down the array of available options, highlighting the advantages and drawbacks of each approach. Simultaneously, further studies will help to validate the applicability of each particular method at both the micro- and the macro-geographical level.

Our results clearly highlighted the clustering of most OXA-23-producing isolates (70%) collected in Rio de Janeiro with EU-II.[7,8,10,11] However, the detection of new STs, together with new allelic profiles determined by SG identification, suggests a local diversity hotspot that is worth exploring.

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Transparency declarations

None to declare.

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Clonal outbreak of Multidrug-Resistant ST92 *Acinetobacter baumannii* in the University Hospital in Pilsen, Czech Republic

Filipa Grosso[1], Sandra Quinteira[2,3,4], Marina Pinheiro[1], Tamara Bergerová[5], Jaroslav Hrabák[5], Luísa Peixe[1]

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

[2]CIBIO. Centro de Investigação em Biodiversidade e Recursos Genéticos. Vairão, Portugal.

[3]Faculdade de Ciências da Universidade do Porto, Departamento de Biologia. Porto, Portugal.

[4]Centro de Investigação em Tecnologias da Saúde (CITS)/IPSN (CESPU), VN Famalicão, Portugal Department of Microbiology, Faculty of Medicine and [5]University Hospital in Pilsen, Charles University in Prague, Pilsen, Czech Republic

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Abstract

Objectives The aim of this study was to characterize the genetic determinants of carbapenem resistance and the molecular epidemiology of multidrug-resistant (MDR) *Acinetobacter baumannii* isolates involved in an outbreak at the University Hospital of Pilsen, Czech Republic.

Methods. Nine non-repetitive isolates representative were selected from a total of 97 clinical isolates collected in different units involved in the outbreak– Cardiosurgery (n=5), Surgery (n=3) and Pulmonary (n=1). These isolates were characterized in what concerns their antimicrobial susceptibility, carbapenem resistance determinants and clonal relatedness by *Apa* I-PFGE, $bla_{\text{OXA-51-like}}$ type identification and two MLST schemes.

Results. Isolates were resistant to all β-lactams, with the exception of one isolate susceptible to meropenem, and to ciprofloxacin. The susceptibility to aminoglycosides was variable and all isolates were susceptible to colistin. Although IS*Aba1* has been found upstream of $bla_{\text{OXA-66}}$ in all isolates, no carbapenemase activity was detected and carbapenem resistance could not be associated with an efflux system. Two isolates revealed the insertion sequence IS*Aba1* disrupting the *carO* gene but presented completely different MICs to carbapenems. PFGE indicated a common pattern, and the two MLST schemes clustered the isolates on ST92/ST2.

Conclusions. Spread of ST92/ST2 *A. baumannii*, a globally disseminated lineage, was responsible for the emergence of carbapenem resistance in this hospital. Particular features of this lineage might contribute for its success and prevalence; however the carbapenem resistance mechanism could not be elucidated.

Multidrug-resistant (MDR) *Acinetobacter baumannii* has emerged as a major nosocomial infectious agent. Resistance to carbapenems, tigecycline and colistin is of particular concern, decreasing the therapeutic options for the treatment of *A. baumannii* infections. Carbapenem resistance has been mainly attributed to the production of carbapenem hydrolysing class D β-lactamases (CHDLs) (4, 7, 12). Non-enzymatic mechanisms such as penicillin-binding proteins (PBPs) modification, overexpression of efflux pumps like AdeABC, and the decreased expression of some porins, may play an important role in the resistance to this antibiotic, both alone or acting in synergy with β-lactamases. For instance, the inactivation of *carO* gene, involved in the OM permeation of L-ornithine, a basic amino acid that displays structural similarity with carbapenems, have been associated with resistance to this class of antibiotics (8).

Since 2003 was observed a rise on *A. baumannii* resistant to carbapenems in several hospitals from Czech Republic. This emergence was associated with the spread of *A. baumannii* strains of European clone II, belonging to sequence type (ST) 2 and ST47 according to Acinetobacter MLST scheme from Institut Pasteur, with isolates carrying $bla_{\text{OXA-58-like}}$, $bla_{\text{OXA-24-}}$like or IS*Aba1* adjacent to $bla_{\text{OXA-51-like}}$ (9).

At the University Hospital in Pilsen, carbapenem resistant *A. baumannii* isolates were firstly identified in 2005, but by the end of 2007 a dramatic increase in infections caused by multidrug-resistant (MDR) *A. baumannii* was observed in different units.

In this study we investigate the clonal relatedness and carbapenem resistance determinants of MDR *A. baumannii* isolates recovered during this outbreak.

In the beginning of 2008, 1-month survey of carbapenem resistant strains of *A. baumannii* was performed at the University Hospital in Pilsen, Czech Republic. From a total of 97 isolates collected during this period, nine non-repetitive multidrug-resistant isolates were selected from different units involved in the outbreak– Cardiosurgery (n=5), Surgery (n=3) and Pulmonary (n=1) (Table 1). Seven of them were associated with clinically manifested infections. Isolates identification was performed with the

Vitek 2 system and confirmed by16S rRNA gene sequencing and by the detection of *bla*$_{\text{OXA-51-like}}$ (6,11).

Minimum Inhibitory Concentrations (MICs) to imipenem and meropenem were performed with E-test (AB-Biodisk, Sweden). MICs to gentamicin, amikacin, ciprofloxacin, and colistin were determined by standard microdilution broth method. Results were interpreted according to EUCAST recommendations (http://www.eucast.org/clinical_breakpoints/).

MBLs genes (*bla*$_{\text{IMP}}$ and *bla*$_{\text{VIM}}$) and CHDL genes (*bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-24-like}}$, *bla*$_{\text{OXA-58-like}}$, and *bla*$_{\text{OXA-143}}$) were sought by PCR (3, 12). Carbapenemase activity was investigated by a bioassay method and by spectrophotometry (4). Imipenem susceptibilitiy in the absence and presence of one-half of the MIC for the efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was examined in all isolates (Shi, 2005).

Sequence analysis of *carO* gene was performed after amplification with primers described by Mussi *et al*, 2011 (8) in three representative isolates (RC-Ab1, RC-Ab2 and RC-Ab6; Table 1).

Pulsed-field gel electrophoresis (PFGE) was performed with the *Apa*I restriction enzyme, and pattern analysis was conducted with InfoQuestTM FP v5.4 (BioRad Laboratories, Hercules, CA, USA). Isolates clustering together with >85% similarity levels were considered to belong to the same PFGE type. Sequence-based typing of *bla*$_{\text{OXA-51-like}}$ gene was performed as previously described (7) and the presence of IS*Aba1* upstream this gene was sought by PCR (10) and confirmed by sequencing. MLST analysis was conducted according to two distinct schemes that will be referred from now on as MLST1, developed by Bartual *et al.*, 2005 (1) and MLST2, published by Nemec *et al,* 2008 (9).

Isolates were resistant to ciprofloxacin and carbapenems with exception of one isolate (RC-Ab1) that was susceptible to meropenem (MIC=2 mg/L). The susceptibility to

aminoglycosides was variable and all isolates were susceptible to colistin. The main characteristics of *A. baumannii* isolates used in this study are summarized in Table 1.

PFGE indicated related patterns and computer-assisted analysis revealed similarity values >95% for the studied isolates.

MLST1 and MLST2 schemes clustered the isolates in the ST92 and ST2 respectively, which correspond to worldwide disseminated lineages (5, 7).

The isolates presented the naturally occurring $bla_{\text{OXA-66}}$ gene with the insertion sequence IS*Aba1* located upstream this gene. We did not observed any known acquired carbapenemase genes, which was confirmed by the absence of carbapenemase activity investigated both by bioassays and spectrophotometry.

It was not observed any reduction in MIC values after the addition CCCP excluding the role of protonic efflux pumps overexpression in the carbapenem resistance of these isolates (10).

PCR amplification with *carO*-specific primers yielded the expected fragments of ca. 750 bp for all isolates except for RC-Ab1 and RC-Ab4 that displayed a fragment with ca 1900 bp. Sequence analysis of *carO* gene for RC-Ab2 and RC-Ab6, taken as representative of carbapenem-resistant isolates, revealed the absence of stop codons. On the other hand, for RC-Ab1 isolate, which displayed lower carbapenem MIC values, *carO* gene was disrupted by the insertion sequence *IS*Aba1 at the nucleotide 462. This way, carbapenem resistance could not be attributed to alterations observed on *carO* nucleotide sequence. It is worth to note that in the majority of reports referring *carO* disruption as being responsible for non-susceptibility to carbapenems the analysed isolates also presented additional resistance mechanisms, such as acquired carbapenemases or efflux pumps overexpression. In addition, reports referring *carO* gene alterations, even in the absence of carbapenemase activity, as being associated with carbapenem resistance, did not investigate the role of other non-enzymatic mechanisms (Mussi, 2005).

In summary, our results confirm the current dominance and global dissemination of the ST92/ST2 lineage and its association with the increasing of multi-drug resistant *A. baumannii* infections observed in several wards of University Hospital in Pilsen. Some studies suggest that the presence of IS*Aba1* upstream $bla_{\text{OXA-66}}$ might be responsible for the carbapenem resistance, but it was not observed any carbapenemase activity in these isolates. Carbapenem resistance could not be attributed to alterations observed in the *carO* gene. Probably an additional unidentified resistance mechanism was the main responsible for the resistance observed to carbapenems.

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Table 1. Characteristics of *A. baumannii* isolates

				Clonality analysis				Antimicrobial susceptibility pattern – MIC (mg/L)					
Isolate	Age	Department	Specimen	PFGE	MLST1	MLST2	OXA-51-type	IPM	MEM	CIP	AMK	GEN	COL
RC-Ab1	63	Surgery - ICU1	bronchoalveolar lavage	A	ST92	ST2	OXA-66	4	2	4	4	1	0.5
RC-Ab2	73	Cardiosurgery - ICU	sputum	A	ST92	ST2	OXA-66	>32	16	16	4	0.25	0.5
RC-Ab3	73	Cardiosurgery - ICU	urine	A	ST92	ST2	OXA-66	>32	16	16	4	0.25	0.5
RC-Ab4	69	Cardiosurgery - ICU	sputum	A	ST92	ST2	OXA-66	>32	32	16	4	16	0.5
RC-Ab5	78	Cardiosurgery - ICU	sputum	A	ST92	ST2	OXA-66	8	16	16	2	32	0.5
RC-Ab6	71	Cardiosurgery - ICU	sputum	A	ST92	ST2	OXA-66	8	16	16	8	32	0.5
RC-Ab7	62	Surgery - ICU2	wound	A	ST92	ST2	OXA-66	>32	8	8	16	16	0.5
RC-Ab8	59	Surgery	wound	A	ST92	ST2	OXA-66	8	16	8	32	>32	0.5
RC-Ab9	73	Pulmonary	catheter	A	ST92	ST2	OXA-66	8	8	16	4	8	0.5

IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; AMK, amikacin; GEN, gentamicin; COL, colistin.

Emergence of an extreme-drug-resistant (XDR) *Acinetobacter baumannii* carrying bla_{OXA-23} in a patient with acute necrohaemorrhagic pancreatitis.

Filipa Grosso[1], Sandra Quinteira[1,2], and Luísa Peixe[1]

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

[2]Centro de Investigação em Tecnologias da Saúde (CITS)/IPSN (CESPU), Vila Nova de Famalicão, Portugal

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Emergence of an extreme-drug-resistant (XDR) *Acinetobacter baumannii* carrying bla_{OXA-23} in a patient with acute necrohaemorrhagic pancreatitis

Madam,

Acinetobacter baumannii is an opportunistic pathogen often associated with serious nosocomial infections and, more recently, with community-acquired pneumonia.[1–3] Although carbapenems and aminoglycosides have been traditionally used in the treatment of these infections, resistance to these antibiotics is now a common occurrence, with Portugal presenting a leading position in terms of incidence rates.[1–3] The acquisition of OXA-type carbapenemases (OXA-23, OXA-40 and OXA-58) is the main documented carbapenemase resistance mechanism, with numerous outbreaks occurring in hospitals worldwide.[1–4] The few remaining therapeutic options include tigecycline and 'old' polimyxins, despite previous withdrawal because of reports of nephrotoxicity and neurotoxicity. Even with these therapeutic alternatives, an increasing recovery of multidrug-resistant (MDR) *A. baumannii* isolates has been observed.[1–3,5] As recently predicted, the emergence of an *A. baumannii* isolate with resistance to all available antibiotics (extreme drug resistance: XDR) is considered an alarming occurrence.[5]

Four *A. baumannii* isolates were recovered from a 37-year-old woman with a history of alcohol abuse, admitted, in 2006, in the emergency ward of the Hospital S. Teotónio, Viseu, Portugal, with acute necrohaemorrhagic pancreatitis. Therapy was initiated with meropenem and fluconazole on the day of admission. Nine days after meropenem administration, *Enterococcus faecium*, *Enterococcus casseliflavus* and *A. baumannii* (HST1) isolates were recovered from intraperitoneal fluid. Another *A. baumannii* isolate (HST1a) was obtained from blood samples, revealing an identical susceptibility profile to that of HST1, including resistance to all available antibiotics, except amikacin. Therapy was replaced by a combination of ampicillin and amikacin, for 19 days, maintaining prophylaxis with fluconazole. Nine days after changing therapy a pancreatic abscess was drained. Cultures of the intraperitoneal fluid revealed *A. baumannii* colonies (HST2 and HST3) presenting not only an MDR pattern similar to that of HST1, but also resistance to amikacin (Table I). HST2 and HST1 were further characterised. The patient was discharged after 18 weeks of hospitalisation. Identification of the isolates was performed both with the Vitek system and 16S rRNA gene sequencing. A multiplex polymerase chain reaction (PCR) for the carbapenemase genes bla_{OXA-23}-like, bla_{OXA-24}-like, bla_{OXA-51}-like, bla_{OXA-58}-like was conducted, producing positive results for bla_{OXA-23}-like and bla_{OXA-51}-like.[1] The bla_{OXA-23} complete gene was confirmed by PCR and sequencing using the primers OXA-23fwd-5′-ATGAATAAATATTTTACTTGC-3′, OXA-23rev-5′-TTAAATAATATTCAGCTGTTT-3′. The association of the bla_{OXA-23} gene with the composite transposon Tn2006 was determined by PCR and a chromosomal location for this gene was observed by I-CeuI technique.[1] HST1 and HST2 revealed the same pulsed-field gel electrophoresis pattern, suggesting the in-vivo acquisition of resistance to amikacin during therapy. Even though the main aminoglycoside resistance mechanism in *A. baumannii* usually relates to integron inserted genes coding for aminoglycoside-modifying enzymes, these isolates revealed integrons with ~500 bp incompatible with the presence of gene cassettes. Detection of genes coding both for aminoglycoside-modifying enzymes and for 16S methylase Arm A was also negative.[1] PCR amplification and sequencing of the regulatory genes for the AdeABC efflux pump, *ade*S and *ade*R, were performed for HST1 and HST2 isolates, revealing the *ade*R substitutions already associated with overexpression of the efflux pump, namely in the residues 120 (isoleucine), 142 (leucine) and 158 (histidine).[2] Reverse transcription–PCR assays for *adeB* gene expression were performed using 16S RNA as a housekeeping gene, and *A. baumannii* ATCC 19606 as a control strain. The results support the overexpression of AdeABC efflux pump, which might explain the observed tigecycline resistance. However, these overexpression levels were identical for both HST1 and HST2 isolates, which does not allow the association of the efflux pump overexpression with the acquired resistance to amikacin.[2] Also, further assays with the protonic efflux pump inhibitor carbonyl cyanide-*m*-chlorophenylhydrazone, CCCP [16 mg/L; corresponding to a quarter of minimum inhibitory concentration (MIC)], did not demonstrate any effect on amikacin MIC levels.

Table I
Antimicrobial susceptibility patterns of *A. baumannii* HST1 and HST2 clinical isolates

Antibiotic	MIC (mg/L)[a]	
	HST1	HST2
Amoxicillin	>256	>256
Amoxicillin–clavulanic acid	>256	>256
Ticarcillin	>256	>256
Ticarcillin–clavulanic acid	>256	>256
Piperacillin	>256	>256
Piperacillin–tazobactam	>256	>256
Ceftazidime	64	64
Cefotaxime	>256	>256
Cefpirome	>256	>256
Cefpime	>32	>32
Cefoxitin	>256	>256
Cefalotin	>256	>256
Imipenem	>32	>32
Meropenem	>32	>32
Colistin	32	32
Minocyclin	32	32
Tigecycline	16	32
Amikacin	16	64

[a] Minimum inhibitory concentrations (MICs) of β-lactams were determined by the Etest method; for colistin, minocycline, tigecycline and amikacin the agar dilution method was applied.[7]

Considerable debate on the proper terminology to be applied to the degree of multidrug resistance in *A. baumannii* is still ongoing. Whether called pandrug, extreme or extensively drug resistant, it is crucial to differentiate strains that are truly resistant to all available therapeutic options.[3,5,6] Recent reports of truly XDR *A. baumannii* strains include OXA-23-producing isolates in South Korea, several isolates from Spain, and one in the USA.[3,4,6] Here, we show the in-vivo evolution of an MDR *A. baumannii* isolate into an XDR, after resistance enhancement to amikacin. This is the first description of OXA-23 carbapenemase in Portugal, which may contribute to an increase in the frequency of isolation of carbapenem-resistant *A. baumannii* isolates. Our results also highlight the inactivity of colistin and tigecycline, recently used as the last therapeutic options for MDR *A. baumannii* infections. The emergence of XDR isolates, in widely disparate regions, is worrying and suggests a spreading pattern that will force public health agents to think globally.

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F. Grosso[a]
S. Quinteira[a,b]
L. Peixe[a,*]

[a]*REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal*

[b]*Centro de Investigação em Tecnologias da Saúde (CITS)/IPSN (CESPU), VN Famalicão, Portugal*

* Corresponding author. Address: Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164 4050-047 Porto, Portugal.
Tel.: +351 222078946; fax: +351 222003977.
E-mail address: lpeixe@ff.up.pt (L. Peixe)

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Effectiveness of podcasts as an adjunct learning strategy in teaching clinical microbiology among medical students

Madam,

The use of information and communication technologies is an increasingly important aspect of medical education, with many universities offering a variety of web-based teaching resources.[1] Educational podcasts, which are audio or visual recordings of items such as lectures, are increasing in number. The advantage of podcasts is that they are easy to use, can be accessed at any time and carry the potential for adding new collaborative dimensions to educational tools for students.

As podcasts are an emerging area of medical education, their true potential is unknown. Whereas there are studies which have assessed students' experience and response to podcasts, there are no studies which assess students' knowledge of selected topics before and after the release of podcasts.[1–3] We aimed to assess the impact of podcasts as an educational tool by ascertaining whether podcasts have a positive impact on students' knowledge of key areas of a curriculum when described in a podcast.

A total of 260 third-year medical students in the Royal College of Surgeons in Ireland studying clinical microbiology were asked to participate in the study, which was carried out from September 2008 to May 2009. A series of 18 podcasts were recorded on individual microbiology topics, e.g. antibiotic treatment of selected infections, prevention of healthcare-associated infections, etc. Topics selected were those which the department felt were important to emphasise, and formed an adjunct to didactic lectures and tutorials. Each podcast, which was developed as an audio podcast, lasted 5–10 min in duration and was recorded using Camtasia™ (TechSmith, Okemos, MI, USA) and subsequently placed on our course management website, Moodle, which allowed students to listen to or download the podcast.

A short online quiz incorporating 10 multiple choice questions relating to points covered in the podcast was devised for each podcast. It was made clear to the students that although the quizzes were not compulsory the department expected the students to partake in the study and avail of the podcasts as an additional educational tool. Over the nine-month period the podcasts and quizzes were released one week following completion of lectures and tutorials on the topic. Initially students completed a quiz relating to information contained in the podcast one day before the release of the podcast. The quiz was then closed and the podcast on the topic released. Following release of the podcast, students performed the same quiz five days later. The number of students completing the quizzes and the quiz results were then recorded and analysed (Student's *t*-test, Microsoft Excel).

An average of 138 students (range: 74–202), from a class of 260, completed the pre- and post-podcast quizzes. The average score (out of 10) of the 18 quizzes released before the podcast was available was 4.94 (range: 2.5–7.3), whereas the average score of the post-podcast quizzes was 6.0 (range: 4.2–8.5). This represents a statistically significant improvement in the post-podcast quiz score ($P < 0.01$). The percentage difference between pre- and post-podcast quizzes is illustrated in Figure 1. When analysing all quiz results there was an average increase of 18.5% in the post-podcast quiz, and in all but one quiz series there was an increase in the students' scores in the post-podcast quiz.

Studies on the use of podcasts in medical education report a favourable response among students to such alternative methods of course delivery. Pilarski *et al.* found that podcasts aided students to learn course material.[2] Shantikumar *et al.* investigated the perceptions of podcasts among students and concluded that podcasts showed promise as a revision aid that could be incorporated into the undergraduate curriculum.[3] This is the first study to evaluate the educational benefit of podcasts by using an assessment of student knowledge before and after access to a podcast. A limitation of this study was that the assessments and uploading of podcasts by students was not compulsory, which may indicate a bias in our results as only the motivated and enthusiastic students may have taken part in the study. However, we would argue that as there was a statistical improvement in our study, which may have included a disproportionate number of good students, these findings could also be extrapolated to poorer students.

Although podcasts are not designed to replace traditional teaching methods such as lectures and real-time classroom interactions, we believe that podcasts and similar technological advances have a key role to play in enhancing the learning experience for students and that, as we have shown in this study, they increase the students' knowledge of key areas of their course.

II. Contribution *of mobile genetic elements for the dissemination of carbapenem resistance among Acinetobacter spp.*

The characterization of *bla*$_{OXA-24/40}$-carrying plasmids and *bla*$_{IMP-5}$ genetic environments, exploring the role of horizontal gene transfer in the dissemination of carbapenem resistance, was conducted and the collected data is presented and discussed within this chapter.

- Grosso F, Quinteira S, Poirel L, Novais A, Peixe L. 2012. The role of common *bla*$_{OXA-24/40}$-carrying platforms and plasmids in the spread of OXA-24/40 among *Acinetobacter* spp. clinical isolates. *Antimicrobial Agents and Chemotherapy*. 56(7):3969-3972.

- Grosso F, Ramos H, Quinteira S, Peixe L. IMP-5-producing multidrug-resistant *Acinetobacter lwoffii* from a newborn incubator. (*submitted*)

Role of Common *bla*$_{OXA-24/OXA-40}$-Carrying Platforms and Plasmids in the Spread of OXA-24/OXA-40 among *Acinetobacter* Species Clinical Isolates.

Filipa Grosso[1], Sandra Quinteira[1,2,3], Laurent Poirel[4], Ângela Novais[1] and Luísa Peixe[1]

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

[2]Centro de Investigação em Tecnologias da Saúde (CITS)/IPSN (CESPU), Vila Nova de Famalicão, Portugal

[3]Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO), Vairão, Portugal

[4]Service de Bactériologie-Virologie, INSERM U914, Emerging Resistance to Antibiotics, Hôpital de Bicêtre, South-Paris Medical School, K.-Bicêtre, France

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Role of Common *bla*$_{OXA-24/OXA-40}$-Carrying Platforms and Plasmids in the Spread of OXA-24/OXA-40 among Acinetobacter Species Clinical Isolates

Filipa Grosso, Sandra Quinteira, Laurent Poirel, Ângela Novais and Luísa Peixe

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Role of Common $bla_{OXA-24/OXA-40}$-Carrying Platforms and Plasmids in the Spread of OXA-24/OXA-40 among *Acinetobacter* Species Clinical Isolates

Filipa Grosso,[a] Sandra Quinteira,[a,b,c] Laurent Poirel,[d] Ângela Novais,[a] and Luísa Peixe[a]

REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal[a]; Centro de Investigação em Tecnologias da Saúde (CITS)/IPSN (CESPU), Vila Nova de Famalicão, Portugal[b]; Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO), Vairão, Portugal[c]; and Service de Bactériologie-Virologie, INSERM U914, Emerging Resistance to Antibiotics, Hôpital de Bicêtre, South-Paris Medical School, K.-Bicêtre, France[d]

The spread of OXA-24/OXA-40 (OXA-24/40)-producing *Acinetobacter* spp. in the Iberian Peninsula has been strongly influenced by clonal expansion, but the role of horizontal gene transfer has scarcely been explored. $bla_{OXA-24/40}$-carrying plasmids and genetic environments were characterized in representative ($n = 15$) *Acinetobacter* species clinical isolates (obtained between 2001 and 2007) by *Acinetobacter baumannii* PCR-based replicon typing, sequencing, hybridization, and restriction fragment length polymorphism. Besides the identification of $bla_{OXA-24/40}$ within the chromosomes of some isolates, the circulation of common $bla_{OXA-24/40}$-carrying plasmids (30-kb *repA_AB*; 10-kb *aci2*) and genetic backbones among *Acinetobacter* spp. was demonstrated.

Carbapenem resistance in *Acinetobacter* spp. has been mostly associated with the production of OXA-23, OXA-24/OXA-40 (OXA-24/40), and OXA-58 carbapenem-hydrolyzing class D β-lactamases (1, 3, 5–12). Whereas OXA-23- and OXA-58-producing *Acinetobacter baumannii* are widely disseminated (3, 6, 12), OXA-24/40 producers have been reported mainly as endemic in the Iberian Peninsula, where the ongoing spread of OXA-24/40 since 1995 has been explained by clonal expansion (6, 10). In addition, there have been reports of sporadic cases in Italy and France and of outbreaks in the United States (5, 7, 8). Recently, some studies demonstrated the mobilization of $bla_{OXA-24/40}$ by lateral gene transfer events (1, 5, 9, 10). In this study, the characterization of the diversity of OXA-24/40-encoding plasmids and the genetic environment of the $bla_{OXA-24/40}$ genes was conducted in *A. baumannii* and *Acinetobacter haemolyticus* clinical isolates from Portugal.

Fifteen representative OXA-24/40-producing *Acinetobacter* spp. isolates (13 *A. baumannii* and 2 *A. haemolyticus*) were selected from a collection of 157 carbapenem-resistant *Acinetobacter* species clinical isolates from two geographically distant Portuguese hospitals (obtained between 2001 and 2007), (i) Hospital Geral de Santo António (HGSA) and (ii) Centro Hospitalar da Cova da Beira (CHCB) (Table 1) (6). Isolates were identified with the Vitek 2 system and 16S rRNA gene sequencing. Detection of the $bla_{OXA-24/40}$ gene was performed as previously described (6, 10). Antibiotic susceptibility testing was done by Etest and the disk diffusion method following CLSI guidelines (4). Isolates were chosen on the basis of their ApaI pulsed-field gel electrophoresis (PFGE) patterns (4 subtypes arbitrarily designated A [$n = 4$; obtained between 2001 and 2004 from HGSA], A1 [$n = 3$; obtained between 2006 and 2007 from CHCB], A3 [$n = 5$; obtained in 2006 from HGSA], and A4 [$n = 1$; obtained in 2003 from HGSA]) and plasmid content (1 to 3 plasmids ranging from 10 kb to 90 kb). They all belonged to the ST98 lineage (6) according to the multilocus sequence typing (MLST) scheme proposed by Bartual et al. (2) (http://pubmlst.org/abaumannii/). This sequence type (ST) is a double-locus variant of the worldwide-disseminated ST92, which is endemic in Portuguese hospitals and has been identified in other European countries associated with the production of different OXA types (6, 12). Conjugative transfer (solid, 37°C) and transformation by electroporation were conducted in a subset of isolates using *A. baumannii* ATCC 19606, *Escherichia coli* K-12 BM21 (rifampin and nalidixic acid resistant, plasmid free), or *Acinetobacter baylyi* ADP1 as the recipient strain, as described previously (5). Transconjugants and transformants were selected using tryptic soy agar (TSA) plates supplemented with rifampin (100 μg/ml) and/or ampicillin (30 μg/ml) or imipenem (0.5 to 1 μg/ml), as reported. The location (chromosome/plasmid) of $bla_{OXA-24/40}$ was assessed by hybridization of I-CeuI and S1 gels with specific probes ($bla_{OXA-24/40}$, 16S rRNA genes) in the wild-type and transformant strains. Plasmid characterization was further accomplished by the recently proposed PCR-based replicon typing scheme for *A. baumannii* plasmids (AB-PBRT), sequencing and hybridization with replicase (*rep*) probes (3), and restriction fragment length polymorphism (RFLP) (EcoRI, BamHI, or HindIII).

An *in silico* comparative analysis of six fully sequenced and one partially sequenced *Acinetobacter* species $bla_{OXA-24/40}$-carrying plasmids deposited in the GenBank database (pABVA01, pMMCU3, pMMCU1, pMMCU2, pMMD, pAB02, and pMMA2, with the respective accession numbers NC_012813.1, GQ904227.1, GQ342610.1, NC_013506.1, GQ904226.1, AY228470.1, and GQ377752.1) was performed at the NCBI website using the BLAST software and the nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on those sequences, the vicinity of the $bla_{OXA-24/40}$ gene was characterized

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Address correspondence to Luísa Peixe, lpeixe@ff.up.pt.

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TABLE 1 Epidemiology and plasmid characterization of representative OXA-24/40-producing *Acinetobacter* species clinical isolates from Portugal

Species	PFGE subtype (no. of isolates)	Yr(s) of isolation	Hospital[a]	$bla_{OXA-24/40}$ plasmid size(s) (kb)[b] (RFLP profile) (no. of isolates)	*rep* content[b] (plasmid size [kb])	Antibiotic resistance profile[d]
A. baumannii	A (4)	2001–2004	HGSA	**90**, **30 (A)** (3)	***repA_AB* (30)**, *repAci2*, *repAci6*	CAZ, ATM, IPM, MEM, PIP, FEP, CIP, TOB, TET, MIN
		2001–2004	HGSA	**70**, **30** (ND) (1)	*repA_AB* (30), *repAci2*, *repAci6*	CAZ, ATM, IPM, MEM, PIP, FEP, CIP
	A1 (3)	2006–2007	CHCB	**10**[c] **(B2)** (3)	*repA_AB*, ***repAci2* (10)**, *repAci6*	CAZ, ATM, IPM, MEM, PIP, FEP, CIP
	A3 (5)	2006	HGSA	**90**, **10 (B2)**[c] (1)	*repA_AB*, ***repAci2* (10)**, *repAci6*	CAZ, ATM, IPM, MEM, PIP, FEP, CIP, (TOB)
		2006	HGSA	**30 (A1)** (4)	***repA_AB* (30)**, *repAci2*, *repAci6*	
	A4 (1)	2003	HGSA	**30** (ND), **10 (B)**[c] (1)	*repA_AB*, ***repAci2* (10)**	CAZ, ATM, IPM, MEM, PIP, FEP, CIP, MIN
A. haemolyticus	(2)	2002	HGSA	**90**, **30 (A1′)**[c] (2)	***repA_AB* (30)**, *repAci2*	IPM, MEM, PIP, CIP, TOB, TET

[a] HGSA, Hospital Geral de Santo António (Northern region); CHCB, Centro Hospitalar da Cova da Beira (Center region).
[b] Plasmid number, size, type, and *rep* content were determined by RFLP, PCR, and hybridization of S1- and I-CeuI-digested genomic DNA with specific probes ($bla_{OXA-24/40}$, *repAci2*, *rep_AB*, and *repAci6*). ND, not done. Plasmids harboring $bla_{OXA-24/40}$ appear in bold. Plasmid transfer by transformation is shown as underlined.
[c] The $bla_{OXA-24/40}$ probe additionally hybridized in a chromosomal band.
[d] Parentheses indicate variability of a given resistance determinant. Resistance among transformants is represented by underlining. Abbreviations: CAZ, ceftazidime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; PIP, piperacillin; FEP, cefepime; CIP, ciprofloxacin; TOB, tobramycin; TET, tetracycline; MIN, minocycline.

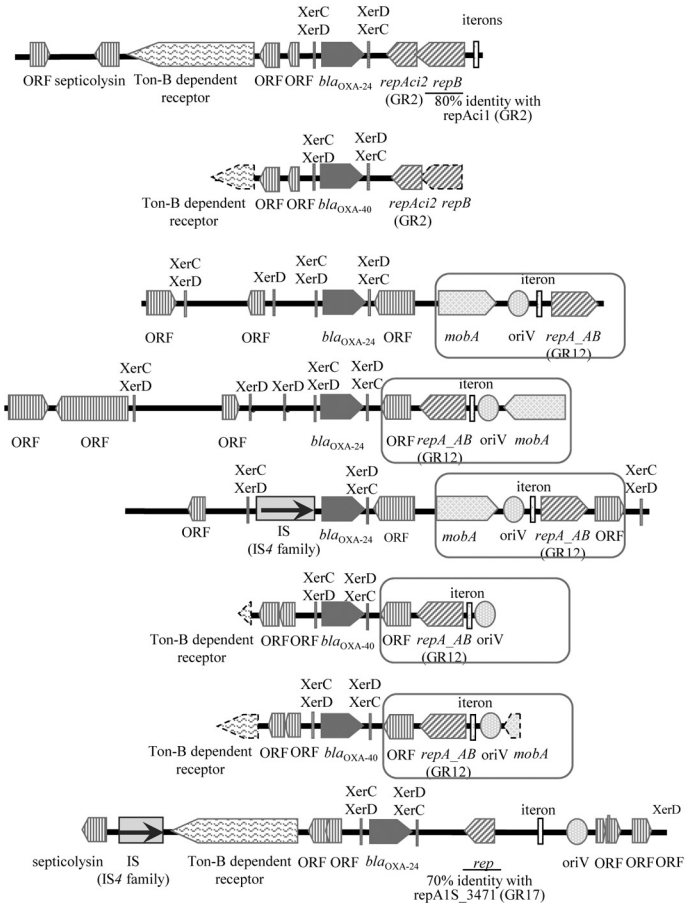
in representative plasmids from our collection by a PCR mapping strategy and further sequencing (Fig. 1).

A. baumannii isolates were resistant to almost all β-lactams, showing variable susceptibility to aztreonam, whereas *A. haemolyticus* isolates ($n = 2$) exhibited resistance to all β-lactams except cefepime, ceftazidime, and aztreonam. All isolates were resistant to ciprofloxacin, whereas susceptibility to aminoglycosides and tetracycline was variable, according to CLSI guidelines (Table 1) (4). As consistently observed in other studies, attempts to obtain OXA-24/40-producing transconjugants were unsuccessful (9). Transformants were obtained for *A. baumannii* isolates belonging to PFGE type A ($n = 1$) and PFGE type A4 ($n = 1$) and one *A. haemolyticus* isolate by using *A. baylyi* ADP1 as the receptor strain, and they acquired resistance to all β-lactams except ceftazidime, aztreonam, and cefepime. Resistance to other groups of antibiotics was not cotransferred. Variable plasmid size (90 kb, 70 kb, 30 kb and/or 10 kb) and *rep* content (GR2 group *aci2* and/or *aci6* and/or GR12 group *repA_AB*) were observed among the different species and PFGE types. The $bla_{OXA-24/40}$ gene was identified within distinct plasmids (70 to 90 kb and 30 kb, 90 kb and 10 kb, 30 kb, or 10 kb) and additionally within the chromosomes of some *A. baumannii* and *A. haemolyticus* isolates (Table 1).

Highly related 30-kb $bla_{OXA-24/40}$-carrying plasmids (arbitrarily designated RFLP pattern A) were identified in *A. baumannii* (PFGE subtypes A and A3) and *A. haemolyticus* isolates collected within the same hospital (obtained between 2001 and 2006) (Table 1). These plasmids belonged to the GR12 group (3) and showed *rep* content (100% identity to *repA_AB*) and sequences surrounding $bla_{OXA-24/40}$ identical to those of the partially sequenced prototype pAB02 plasmid (GenBank accession number AY228470.1) (Fig. 1). A module consisting of an open reading frame (ORF) of unknown function, the replicase *repA_AB*, *oriV*, and *mobA* (represented within a rectangle in Fig. 1) was also found within that structure. Interestingly, it has been previously identified in other 10-kb *repA_AB* plasmids and seems to be a recurrent platform in plasmids (10 kb/30 kb) from different *A. baumannii* clones and *Acinetobacter* species in several European countries (Portugal, Spain, and France) (Fig. 1) (1, 9). The presence of *mob* genes probably facilitates plasmid mobilization by self-transmissible plasmids and may ultimately explain their occurrence in non-*A. baumannii* species (3, 9, 10).

A 10-kb OXA-24/40-encoding plasmid (arbitrarily designated RFLP pattern B) was also frequently identified among *A. baumannii* isolates (PFGE subtypes A1, A3, and A4) collected from different hospitals (obtained between 2003 and 2007) (Table 1). This plasmid belonged to the GR2 group (3), with a *rep* sequence identical to that of *aci2* and a $bla_{OXA-24/40}$ genetic environment closely related to those from the pABVA01 and pMMCU3 plasmids (GenBank accession numbers NC_012813 and GQ904227.1, respectively) identified in *A. baumannii* isolates from Italy and Spain, respectively (1, 5, 9).

The same *rep* genes (GR2, GR6, and GR12) have been presumptively associated with other OXA-24/40-producing *A. baumannii* plasmids from Portugal and Spain by AB-PBRT, thus suggesting the circulation of a common plasmid pool (3, 12). Both $bla_{OXA-24/40}$ genes and larger contiguous modules are flanked by XerC/XerD-like binding sites (Fig. 1), which might be responsible for their mobilization (5, 9) and may explain the identification of $bla_{OXA-24/40}$ and/or common modules in different plasmid scaffolds or in chromosomes. Furthermore, the variability observed in the number and location of the *orf* of unknown function, *oriV*, and iteron sequences suggests frequent recombinatorial events (5, 9). This study not only confirms the horizontal transmission of common resistance modules between *Acinetobacter* plasmids but also highlights interspecies plasmid transfer. Although conjugative transfer was not detected, the *aci6* replicase (GR6; 100% identity to that of plasmid pACICU2 [GenBank accession number NC_010606]) was able to mediate horizontal transfer of antibiotic resistance plasmids, as previously suggested (3, 12). Moreover, a recently described mechanism involving the release of outer membrane vesicles harboring $bla_{OXA-24/40}$-carrying plasmids might also shed some light on this process (11). It is interesting to highlight that the $bla_{OXA-24/40}$ genetic environment and plasmid types are different from those observed for bla_{OXA-23} or bla_{OXA-58}, thus suggesting independent acquisition and subsequent selection events (3, 12).



Plasmid (Accession number)	Size (bp)	Species (Country)	Date	Sequence type	Reference(s)
pABVA01 (NC_012813)	8963	*A. baumannii* (Italy)	2000	Unknown	5
pMMCU3 (GQ904227.1)		(Spain)	2006-2008	Unknown	1, 9
pAC92	~10000 (partial sequence)	*A. baumannii* (Portugal)	2003-2007	ST98	This study
pMMCU1 (GQ342610.1)	8771	*A. calcoaceticus* (Spain)	2006-2008	Unknown	1, 9
pMMCU2 (NC_013506.1)	10270	*A. baumannii* (Spain)	2006-2008	Unknown	1, 9
pMMD (GQ904226.1)	9964	*A. baumannii* (Spain)	2006-2008	Unknown	1, 9
pAB02 (AY228470.1)	4162 (partial sequence)	*A. baumannii* (France)	(2004)	Unknown	Unpublished
pAC63	~30000 (partial sequence)	*A. haemolyticus* *A. baumannii* (Portugal)	2001-2006	NA ST98	This study
pMMA2 (GQ377752.1)	10679	*A. baumannii* (Spain)	2006	ST56	1, 9

FIG 1 $bla_{OXA-24/40}$ genetic surroundings observed in clinical *Acinetobacter* spp. from different countries that were identified in this and previous studies. Parentheses around a year indicate that the year of submission to GenBank is given because the isolation date is unknown. Discontinuous lines represent partial sequences. NA, not applicable; ORF, open reading frame.

In this study, common $bla_{OXA-24/40}$-carrying plasmids (30-kb *repA_AB* and 10-kb *aci2*) were identified among representative ST98 *A. baumannii* and *A. haemolyticus* clinical isolates collected over a large period of time (2001 to 2007). In addition, with a systematic *in silico* analysis, we were able to identify the same plasmid types and closely related $bla_{OXA-24/40}$ genetic surroundings in OXA-24/40-producing isolates from different European countries. These findings, together with some previous evidence for horizontal gene transfer of $bla_{OXA-24/40}$-carrying modules, strongly supports the role of common platforms and plasmids in the dissemination process of OXA-24/40 among *Acinetobacter* spp.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study that correspond to the representative 30-kb *repA_AB* (pAC63) and 10-kb *aci2* (pAC92) OXA-24/40-encoding plasmids have been submitted to the GenBank database (GenBank accession numbers JN982951 and JN982952, respectively).

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IMP-5-producing multidrug-resistant *Acinetobacter lwoffii* from a newborn incubator

Filipa Grosso[1], Helena Ramos[2], Sandra Quinteira[3,4,5], Luísa Peixe[1*]

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

[2] Hospital Geral de Santo António, Porto, Portugal.

[3] CIBIO. Centro de Investigação em Biodiversidade e Recursos Genéticos. Vairão, Portugal.

[4.] Faculdade de Ciências da Universidade do Porto, Departamento de Biologia. Porto, Portugal.

[5.] Centro de Investigação em Tecnologias da Saúde (CITS)/IPSN (CESPU), VN Famalicão, Portugal

Manuscript submitted

Sir,

The survival chances of premature infants have remarkably increased with the development of incubators. Nevertheless, the thermoneutral environment provided by these devices increases the risk of microbial infection, which is also associated with the growing proportion of very low birth weight neonates and with the invasive diagnostic/therapeutic procedures they undergo. As a result, nosocomial infections remain important and critical issues related to high morbidity and mortality in high-risk neonates, a very unique population that naturally present immaturity of immune system and impaired defense mechanisms (8).

Most nosocomial infections in neonatal intensive care units (NICU) have been attributed to *Acinetobacter* species, which surprisingly and successively evolved, within the last decades, from traditionally harmless organisms into important nosocomial pathogens (5, 6, 8). Additionally, the spread of multidrug resistant strains became a worldwide threat and, with extremely limited therapeutic options, carbapenems emerged as last resource to treat *Acinetobacter*-causing infections. Unfortunately, *A. baumannii* strains resistant to all known antibiotics have now been reported and carbapenem resistance within this genus is now a global phenomenon (6).

Acinetobacter species other than *A. baumannii*, although less frequently reported in neonatal population, are also known to play a role within the clinical setting (5, 7-9). In fact, its prevalence as etiologic agents of nosocomial infections has increased in last years, as demonstrated by *Acinetobacter lwoffii*, a commensal organism found on human skin, perineum and oropharynx. This species has been associated with catheter-related bloodstream infections in immunocompromised patients and with bacteremia associated with community-acquired gastroenteritis and gastritis (9).

Both the increased resistance to irradiation observed for *A. lwoffii* (which raises concerns about the efficacy of sterilization of medical devices by this method) together with the species ability to acquire foreign genetic determinants might partially explain the current emergence of this new opportunistic pathogen (9). Recently, Figueiredo *et al* demonstrated that *A. lwoffii* intrinsically possesses a chromosomal gene encoding a CHDL, OXA-134-like, and concluded that this species could therefore constitute a reservoir for carbapenemase genes that may spread among other *Acinetobacter* species (5). Besides this naturally occurring gene, which nevertheless does not confer resistance to carbapenems, a recent report of multi-drug resistant *A. lwoffii* clinical isolates harboring $bla_{\text{NDM-1}}$-bearing plasmids illustrates the clinical role that this species might play in the near future (7). Anyhow, as far as we know, no other carbapenem resistance determinant has been yet reported for *A. lwoffii*.

In this report, we describe and characterize an *A. lwoffii* isolate, obtained from a newborn incubator's humidity chamber, which drew our attention by its unusual multi-drug resistance phenotype, which includes resistance to carbapenems.

In June of 2008, a newborn kept in an incubator at a University Hospital in Porto, Portugal, presented a feverish condition. Environmental samples obtained from the water reservoir associated with the incubator´s humidity system led to the detection of an *A. lwoffii* isolate (strain HGSA93). Identification was performed with the Vitek 2 system and confirmed by 16S rRNA gene sequencing and by the presence of $bla_{\text{OXA-134-like}}$ (5). HGSA93 revealed an unusual multi-drug resistance pattern, including resistance to carbapenems (imipenem and meropenem MICs >32mg/L). Antibiotic susceptibility testing was performed by disc diffusion method and E-test (2), revealing that HGSA93 was resistant to other β-lactams, including amoxycillin (>256 mg/L), amoxycillin/clavulanate (32 mg/L), piperacillin/tazobactam (32 mg/L), ceftazidime

(>256 mg/L), cefepime (>32 mg/L), aztreonam (32 mg/L), but remained susceptible to aminoglycosides (except kanamycin), minocycline and tigecycline. A bioassay conducted with the isolates' crude extracts, incubated with imipenem (with and without EDTA), strongly suggested the presence of a MBL, identified as *bla*$_{\text{IMP-5}}$ after PCR (3) and sequencing. Isoelectric focusing only showed a band with a pI compatible with IMP-5 (3). The chromosomal location of the MBL gene was assessed by hybridization assays. PCR mapping showed the association of *bla*$_{\text{IMP-5}}$ gene with In*76* class 1 integron, embedded in a Tn*402*-like transposon, as previously described (4), emphasizing the possibility of inter-specific transference. Curiously, sequence analysis indicated the presence of a weak -35 promoter (TTGACA instead of TTGATA) (1), contrarily to what was expected by the high carbapenem resistance level observed in HGSA93.

IMP-5 has only been described so far in Portugal, initially in an *A. baumannii* clinical isolate belonging to the sequence type (ST) 120 (3) and posteriorly in *Pseudomonas aeruginosa* clinical isolates (1). Notwithstanding, the reservoir of this "exclusively Portuguese" *bla*$_{\text{IMP-5}}$ gene is yet to be known.

It is unclear whether the *bla*$_{\text{IMP-5}}$-carrying *A. lwoffii* strain of environmental origin identified in this study represents a sporadic case or the early stage of a wide dissemination trend. Therefore, we believe that further studies focused on the local microbial ecology of carbapenemase-producers are imperative for the full understanding of the observed endemicity of *bla*$_{\text{IMP-5}}$ in our country and for the effective prevention of its dissemination.

In this report, *A. lwoffii* was not associated with newborn infection, however its detection in the newborn incubator's humidity chamber should warn to the possibility of "environmental" species emerge as etiologic agents of infection within vulnerable

populations, or more worryingly, as reservoir/source of genetic determinants of antibiotic resistance. Recently, *A. lwoffii* was associated to severe infections in infants at a NICU with high mortality rate, as only one case was successfully treated with imipenem, while three cases died from severe ventilator-associated pneumonia, and severe sepsis (7).

Our data highlight the potential of non-*baumannii Acinetobacter* species to act as possible reservoirs of clinically relevant resistance genes and to emerge as important pathogens as occurred with *A. baumannii*, thus constituting a challenge for therapeutic management.

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III. *Contribution of adhesion and biofilm production for the persistence of carbapenem-resistant A. baumannii lineages*

In this study the biofilm formation ability of spread carbapenem resistant *A. baumannii* lineages was investigated. The possible role of $bla_{OXA-24/40}$-carrying plasmids as a genetic platform for the anchorage of biofilm formation capabilities, as well as its contribution for the endemicity of some lineages in Portugal, is also discussed.

- Vuotto C, Grosso F, Costa ML, Ferreira H, Quinteira S, Donelli G, Peixe L. Contribution of adhesion and biofilm production in the persistence of carbapenem-resistant *A. baumannii lineages*: exploring the role of $bla_{OXA-24/40}$-carrying plasmids. (*in preparation*)

Contribution of adhesion and biofilm production in the persistence of carbapenem-resistant *A. baumannii*: exploring the role of *bla*$_{OXA-24/40}$-carrying plasmids

Claudia Vuotto[1], Filipa Grosso[2], Maria Luísa Costa[2], Helena Ferreira[2], Sandra Quinteira[3, 4, 5], Gianfranco Donelli[1], Luísa Peixe[2]

[1] Microbial Biofilm Laboratory, IRCCS Fondazione Santa Lucia, Rome, Italy

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

[3] CIBIO. Centro de Investigação em Biodiversidade e Recursos Genéticos. Vairão, Portugal.

[4.] Faculdade de Ciências da Universidade do Porto, Departamento de Biologia. Porto, Portugal.

[5.] Centro de Investigação em Tecnologias da Saúde (CITS)/IPSN (CESPU), VN Famalicão, Portugal

Manuscript in preparation

Abstract

Biofilm formation ability has been associated with persistence of *Acinetobacter baumannii* (AB) in the hospital environment and propensity to cause infection. In this study we investigated the ability to form biofilm of disseminated carbapenem resistant AB (CRAB) lineages and the possibility for this feature to be encoded by $bla_{OXA-24/40}$-carrying plasmids. Adherence and biofilm formation was evaluated in AB clinical isolates (n=25) belonging to the main lineages circulating in Portugal, bla_{OXA-23}-carrying/ST92, bla_{OXA-40}-carrying/ST98, and bla_{OXA-58}-carrying/ST103. Biofilm production was confirmed with ultramicroscopic analysis (CLSM and FESEM). Overall, isolates of globally disseminated AB (CRAB) lineages demonstrated strong adherence properties and ability to form biofilms in abiotic surfaces which might contribute to their emergence and persistence, particularly among those belonging to bla_{OXA-40}-carrying/ST98 lineage. Confirmation of a complex structure compatible with biofilm production was observed by ultramicroscopic analysis in three $bla_{OXA-24/40}$-carrying isolates. Although acquisition of $bla_{OXA-24/40}$-carrying plasmids could contribute to enhance AB virulence, these plasmids do not contribute to improve adherence and biofilm formation in AB.

Acinetobacter baumannii has become in the last decades one of the most troublesome pathogens, particularly in the hospital setting (2, 4, 5-9).

Its persistence has been attributed to a remarkable ability to acquire genetic resistance determinants and capacity to form biofilms. Multidrug- and, in particular, carbapenem-resistant, *A. baumannii* population structure is highly clonal (1, 5). Until now only very few lineages were associated with carbapenem resistance dissemination, probably because besides resistance, they present particular features that confer additional selective advantages. Among them we can find those belonging to the worldwide disseminated clonal complex (CC) 92, represented in Portugal by the *bla*$_{\text{OXA-23}}$-carrying/ST92 and *bla*$_{\text{OXA-40}}$-carrying/ST98 (5). A third lineage, *bla*$_{\text{OXA-58}}$-carrying/ST103, with no apparent relationship with CC92, was also identified. We previously demonstrated that OXA-40, a carbapenem hydrolyzing class D β-lactamase (CHDL), was encoded in plasmids, belonging to GR2 (*repAci2*) and GR12 (*repA_AB*) homology groups (6), but the possibility that other features contributing for this lineage persistence might be encoded on these plasmids has not been explored until now, particularly because they harbor a TonB-dependent receptor gene coding for an outer membrane protein involved in iron uptake and virulence (1, 6).

Biofilms are highly structured communities of bacteria attached to a surface, either biotic or abiotic, and constitute an important requirement for chronic colonization of human tissues and persistence in implanted medical devices (7). Biofilms are associated with multiple drug resistance and to higher resistance to desiccation, environmental stress (nutritional or oxidative stress), UV light exposure and phagocytosis (8, 9).

In this study we investigated the biofilm formation ability of disseminated carbapenem resistant *A. baumannii* (CRAB) lineages, a feature usually accessed by a biomass quantification assay, which only measures adhesion ability. Biofilm production was further confirmed by fluorescence microscopy and field emission scanning electron microscopy. The possibility of this feature being favored by *bla*$_{\text{OXA-24/40}}$-carrying plasmids acquisition was also explored.

From a collection of 213 CRAB clinical isolates were selected 25 CHDL-producing isolates obtained from five Portuguese hospitals (2001-2008). This selection aimed to include in this study isolates with different acquired CHDLs, belonging to distinct sequence types, and collected from different hospitals over the course of several years. Isolates were distributed among three different lineages: *bla*$_{\text{OXA-23}}$–carrying/ST92 (n=5; 2006–2008), *bla*$_{\text{OXA-40}}$-carrying/ST98 (n=12; 2001–2007) and *bla*$_{\text{OXA-58}}$-carrying/ST103 (n=8; 2001-2008), and obtained from different sources (Table 1). ST98 and ST92

belong to the worldwide disseminated CC92, which has been previously associated with European lineage II (6). The study also included two transformants harboring *bla*$_{OXA-40}$-carrying plasmids and the respective receptor strain *Acinetobacter baylyi* ADP1.

Adherence was tested by a biomass quantification assay as described by Donelli *et al*, 2007 (3), with LB Broth cultures incubated in 96-well plates. The optical density (OD) of each well was measured at 570 nm by using an automated spectrophotometer, with each assay being performed in triplicate and repeated 3 times. As a negative control was used a well with fresh LB supplemented with 1% glucose without bacteria and as a positive control for the biofilm phenotype was used the reference strain *A. baumannii* ATCC 19606T. Isolates were classified into the following categories: non-adherent (OD≤ODc), weakly adherent (ODc<OD≤2XODc), moderately adherent (2ODc<OD≤4XODc), strongly adherent (4xODc<OD). The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control.

Biofilm growth for three highly adherent isolates was assessed by fluorescence microscopy and field emission scanning electron microscopy (FESEM). Image analysis was performed by using ImageJ software (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/). Biofilm surface area coverage was measured by image analysis of micrographs taken at a magnification of 10KX and 25KX. The average area of coverage was obtained from five measurements taken at randomly sorted locations over the polymer surface (3).

Most of the studied isolates were classified as strongly adherents, a feature which, in addition to multidrug resistance observed among them, might explain these lineages persistence in Portuguese hospitals. However, it is interesting to note the diversity in adhesion ability observed among clonally related isolates, similar to what was obtained by other studies (4). Among the three different lineages studied, those belonging to CC92 presented higher adhesion values, which is in accordance to what has already been established for isolates belonging to European lineage II (4). On the other hand, *bla*$_{OXA-58}$-carrying/ST103 isolates were those who presented lower adhesion values, particularly the more recent ones (Table 1). These findings together with their susceptibility profile might explain the restricted dissemination of this lineage (5). In what concerns the pandrug resistant isolate (HST2; Table1), it was observed a strong adhesion value, suggesting that resistance acquisition do not interfere with biofilm production, which contrast with observations of fitness reduction as a result of resistance acquisition (8). It is interesting to note that isolates from catheters were the ones who presented higher adhesion values.

Biomass quantification assay results were further confirmed with the observation of complex structures compatible with biofilm production by FESEM: exopolysaccharide, pili and water channels (Figure 2D), were observed in three *bla*$_{OXA-40}$-carrying isolates. Although *bla*$_{OXA-40}$-carrying/ST98 presented higher adherence values we could not associate the presence of *bla*$_{OXA-40}$-carrying plasmids with this feature, since were not observed differences in adhesion values between the *bla*$_{OXA-40}$-transformants and *A. baylyi* ADP1 receptor strain (Figure 2).

In summary, our study confirms the strong ability presented by most of *A. baumannii* isolates to adhere to abiotic surfaces and to produce biofilms, revealing that adhesion values obtained by biomass quantification assays are good predictors of biofilm production. Antimicrobial resistance does not seem to be diminished in isolates with strong ability to adhere and produce biofilm. In addition, *bla*$_{OXA-40}$-carrying plasmids are not associated with this feature.

Further studies involving genetic determinants responsible for these characteristics are needed to better understand the main features that confer selective advantages among these lineages and the mechanisms underlying biofilm production.

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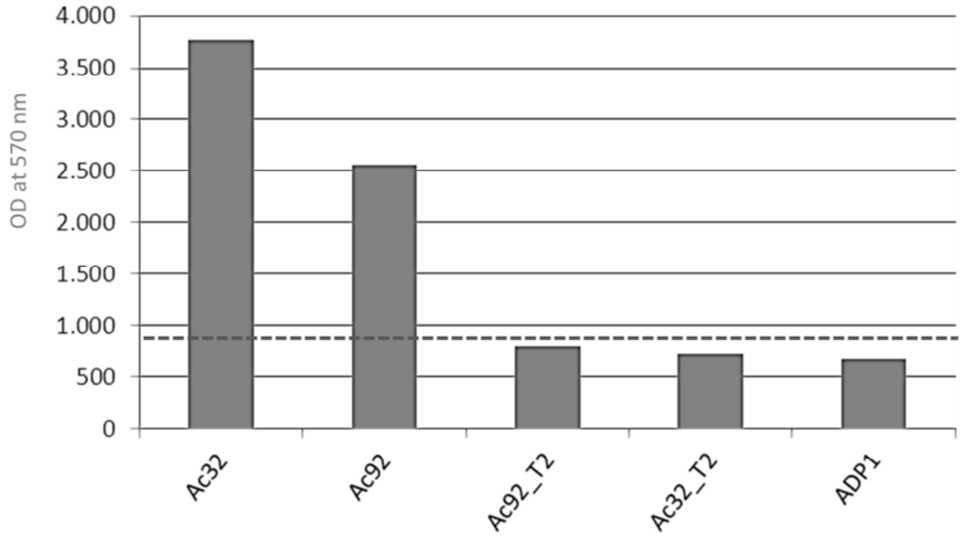

Figure 1. Adhesion values for isolates harboring *bla*$_{OXA-40}$-carrying plasmids, respective transformants, and receptor strain obtained by the biomass quantification assay. The discontinuous line indicates the cut-off above which isolates are classified as strongly adherent (4xODc<OD).

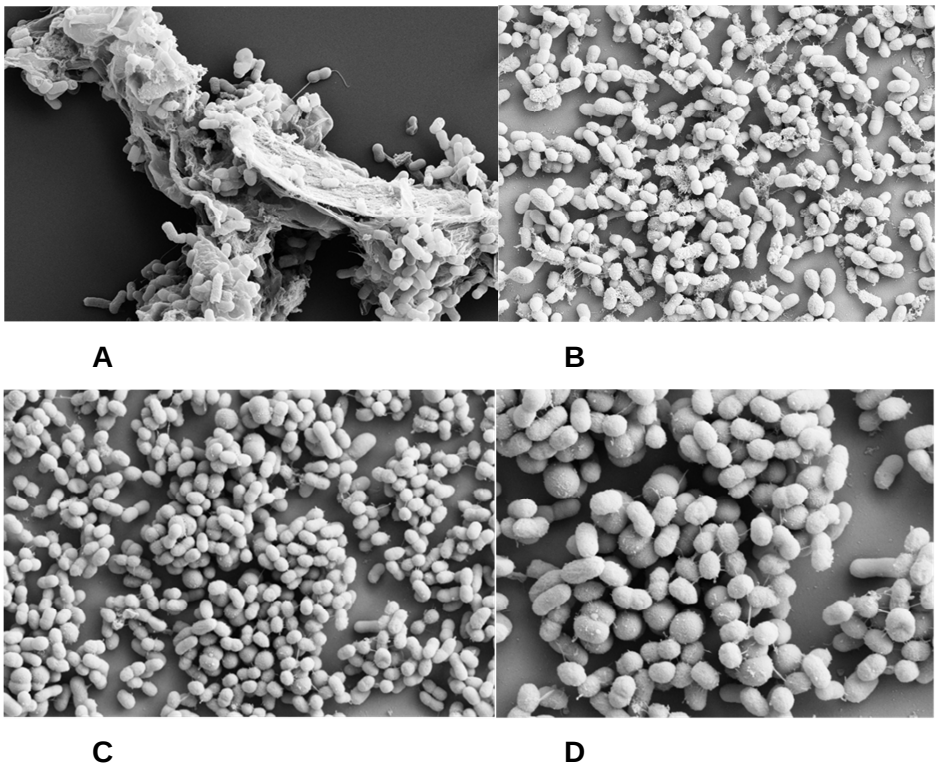

Figure 2. Micrographs of biofilm-producers observed with a magnification of 10.00 KX using an EHT = 5.00 kV: (A) AC92; (B) AC111; (C) HGSA25. D Micrograph of HGSA25 with a magnification of 20.00 KX.

Table 1. Characteristics of the isolates included in the study

Isolate	Year	Hospital[a]	Source[b]	CHDL[c]	PFGE [d]	ST[e]	RFLP/rep[f]	Adhesion[g]
Ac2	2001	HGSA	BS	OXA-40	A	ST98	ND	+++
Ac32	2001	HGSA	BS	OXA-40	A	ST98	A/*repA_AB*	+++
Ac55	2002	HGSA	BS	OXA-40	A	ST98	ND	+
Ac92	2003	HGSA	BS	OXA-40	A4	ST98	B/*repAci2*	+++
Ac111	2003	HGSA	CT	OXA-40	A	ST98	ND	+++
Ac250	2004	HGSA	BS	OXA-40	A	ST98	A/*repA_AB*	+++
HGSA7	2006	HGSA	BS	OXA-40	A3	ST98	A1/*repA_AB*	+++
HGSA8	2006	HGSA	BS	OXA-40	A3	ST98	B2/*repAci2*	-
HGSA11	2006	HGSA	CT	OXA-40	A3	ST98	B2/*repAci2*	+++
HGSA20	2006	HGSA	U	OXA-40	A3	ST98	B2/*repAci2*	+++
HGSA25	2006	HGSA	CT	OXA-40	A3	ST98	B2/*repAci2*	+++
CHCB10	2007	CHCB	U	OXA-40	A1	ST98	B2/*repAci2*	+++
HST1	2006	HST	IPF	OXA-23	A4	ST92	-	+++
HST2	2006	HST	IPF	OXA-23	A4	ST92	-	+++
HPH1	2006	HPH	BS	OXA-23	A4	ST92	-	+++
HA2	2007	HA	INA	OXA-23	A4	ST92	-	+++
HGSA21	2007	HGSA	INA	OXA-23	A4	ST92	-	+++
HST21	2008	HST	CT	OXA-23	A4	ST92	-	+++
Ac23	2001	HGSA	CT	OXA-58	B	ST103	-	+++
Ac42	2001	HGSA	CT	OXA-58	B	ST103	-	+++
Ac67	2002	HGSA	CT	OXA-58	B	ST103	-	+++
Ac70	2002	HGSA	U	OXA-58	B	ST103	-	+++
Ac156	2003	HGSA	CT	OXA-58	C	ST103	-	+++
Ac174	2004	HGSA	U	OXA-58	C	ST103	-	+
Ac246	2004	HGSA	U	OXA-58	B	ST103	-	+
HGSA140	2008	HGSA	BS	OXA-58	B	ST103	-	+
A. baylyi ADP1	-	-	-	-	-	-	-	+
Ac32_T2[h]	-	-	-	OXA-40	-	-	A/*repA_AB*	+
Ac92_T2[h]	-	-	-	OXA-40	-	-	B/*repAci2*	+

[a] HGSA, Hospital Geral de Santo António; CHCB, Centro Hospitalar da Cova da Beira; HST, Hospital de São Teotónio; HPH, Hospital de Pedro Hispano; HA, Hospital de Amarante; [b] BS, bronchial secretions; CT, catheter; U, urine; IPF, intraperitoneal fluid; INA, information not available; [c]CHDL, acquired carbapenem hydrolyzing class D β-lactamases; [d]PFGE, *Apa*I-Pulsed-field gel electrophoresis profile; [e]ST, sequence type determined according to Bartual *et al* (2005) scheme; [f] RFLP, restriction fragment length polymorphism profile (*Eco*RI, *Bam*HI, or *Hind*III)/rep, bla_{OXA-40} carrying plasmid replicase content: *repA_AB* (GR12), *repAci2* (GR2); [g] adhesion classification: +++, strongly adherent; +, weakly adherent; -, non-adherent; [e]; [h] Transformants harboring bla_{OXA-40} carrying plasmid from isolates Ac32 and Ac92.

Chapter 4 SUMMARY CONCLUSIONS

“Experimental science is the queen of sciences and the goal of all speculation.”

Roger Bacon

(1214-1294)

CHAPTER 4 – SUMMARY CONCLUSIONS

The overall results of this thesis evidence that carbapenem resistance in *A. baumannii* results from the interplay of two events: the presence of particular lineages with a great capacity to disseminate and acquire resistance, and the frequent association of carbapenem resistance determinants with mobile genetic elements. This late characteristic also contributes for carbapenem resistance dissemination to other *Acinetobacter* species, which may constitute a widespread and worrisome reservoir of carbapenem resistance genes. In addition, dissemination and persistence of particular carbapenem resistance lineages seems also to depend on virulence factors, such as adhesion and biofilm production, a characteristic that may provide selective advantages to these organisms.

These conclusions result after the fulfillment of the specific objectives of this study:

1. **To characterize the population structure of carbapenem-resistant *Acinetobacter baumannii* clinical isolates collected in the hospital setting in Portugal (1995-2008), Brazil (2006-2007) and Czech Republic (2008).**

   Sequence based typing methods used to analyze population structure of *A. baumannii* clinical isolates revealed that very few lineages are associated with carbapenem-resistance. Remarkably, our data point to higher resolution of Bartual MLST scheme, which provided a better association between epidemiological features, acquired CHDL content and temporal distribution of the studied isolates.

   Very few lineages seem to be contributing for carbapenem-resistance in *A. baumannii*. Our results show the overrepresentation of the CC92 in Portugal, with the current dominance of ST92 carrying $bla_{\text{OXA-23}}$, a lineage also disseminated worldwide. In our country this lineage was able to gradually replace the endemic clone ST98 carrying $bla_{\text{OXA-24/40,}}$ an event only detected by the use of an MLST scheme developed by Bartual *et al* (2005), which provided a better insight on *A. baumannii* population dynamics. An enhanced multidrug resistance profile seems to have contributed to the population switching observed. A third lineage identified corresponds to ST103 carrying $bla_{\text{OXA-58}}$, presenting a lower expression in the context of CRAB lineages, probably due to a more susceptible phenotype. In Czech Republic ST92 also seems to be disseminated and contributing to carbapenem resistance, although not

associated with *bla*$_{OXA-23}$. The identification of new STs among *A. baumannii* clinical isolates from Brazilian hospitals suggests a local diversity hotspot, although was possible to relate them with the main European lineages.

2. **To characterize the carbapenem resistance mechanisms and mobile genetic elements responsible for the spread of carbapenem resistance determinants among *Acinetobacter* species.**

Carbapenem resistance was associated with the presence of carbapenemases, with the exception of Czech Republic's isolates, which demonstrates impairment on CarO porin encoding gene, compromising carbapenems cell entrance. The majority of carbapenemases identified belonged to the group of carbapenem-hydrolyzing class D β-lactamases (CHDLs), thus confirming CHDLs as the main mechanism responsible for resistance to this class of antibiotics. In Portugal were identified OXA-23, OXA-24/40 and OXA-58, and, although associated with mobile genetic elements, was observed stability of a specific acquired CHDL within each lineage. Concerning Czech Republic's isolates, was not possible to elucidate the carbapenem-resistance mechanism, but this finding highlight the possibility that other unidentified resistance mechanisms start to spread in the near future. Only two types of non-conjugative plasmids harbouring *bla*$_{OXA-24/40}$ were identified: 30-kb *repA_AB* (GR12) and 10-kb *repaci2* (GR2) were identified both among *A. baumannii* and *A. haemolyticus* clinical isolates collected over a large period of time (2001 to 2007). This is in accordance to what has been described in other countries, although associated with isolates belonging to different STs, which stresses the contribution of these plasmids in the dissemination process of OXA-24/40.
Moreover, both bla$_{OXA-24/40}$ genes and larger contiguous modules are flanked by XerC/XerD-like binding sites, which might be responsible for their mobilization and may explain the identification of *bla*$_{OXA-24/40}$ and/or common modules in different plasmid scaffolds or in chromosomes. Furthermore, the variability observed in the number and location of the orf of unknown function, oriV, and iteron sequences suggests frequent recombinatorial events on these plasmids. This study not only confirms the horizontal transmission of common resistance modules between *Acinetobacter* plasmids but also highlights interspecies plasmid transfer.

3. **To explore the mechanisms that might drive the antimicrobial resistance evolution in *A. baumannii*.**

   The detection of an *A. baumannii* clinical isolate resistant to all available antibiotics in the course of antibiotic therapy emphasizes the contribution of antimicrobial pressure in the *in vivo* evolution of multidrug-resistant clones into pandrug-resistant ones. This constitutes a concerning event, particularly when occurs within largely disseminated lineages like ST92. In addition, this isolate revealed increased expression of AdeABC efflux pump, possibly associated with the inactivity of tigecycline, a last resource antibiotic for the treatment of MDR *A. baumannii* infections.

4. **To explore the role of environmental *Acinetobacter* species in the acquisition and dissemination of carbapenem resistance mechanisms.**

   The occurrence of an environmental $bla_{IMP\text{-}5}$-carrying *A. lwoffii* pointed for the possible contribution of non-*baumannii Acinetobacter* to act as possible reservoirs of clinically relevant resistance genes and to emerge as important pathogens. Moreover, the observation of $bla_{OXA\text{-}24/40}$ carrying *A. haemolyticus* is of great concern due to the ubiquity of this *Acinetobacter* species.

5. **To characterize the adhesion ability and biofilm formation displayed by the main *A. baumannii* lineages disseminated in Portuguese hospitals.**

   Adhesion ability and biofilm formation together with an enhanced antimicrobial resistance seem to be important features responsible for the persistence and dissemination of the main *A. baumannii* lineages in the Portuguese hospitals. Adhesion abilitie seems do not be favoured by the presence of plasmids of incompatibility group 12 or 2 carrying $bla_{OXA\text{-}24/40.}$ Future studies concerning the genetic basis of these traits will be needed for a better understanding of *Acinetobacter* pathogenesis.

Chapter 5 REFERENCES

Information is a source of learning. But unless it is organized, processed, and available …, it is a burden, not a benefit.
William Pollard
(1911-1989)

CHAPTER 5 - REFERENCES

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Chapter 6 APPENDIX

“Measure what is measurable, and make measurable what is not so.”
Galileo Galilei
(1564-1642)

CHAPTER 6 - APPENDIX

6.1 MATERIAL AND METHODS

6.1.1 Bacterial isolates and epidemiological background

The collection of *Acinetobacter* spp isolates (n=265, corresponding to *A. baumannii*, n=262; *A. haemolyticus,* n=2; and *A. lwoffii*, n=1) included in this thesis was obtained between 1995 and 2008. This collection included previously published isolates obtained between 1995 and 2003 (*A. baumannii*, n=8; *A. haemolyticus*, n=2), with the purpose of plasmid characterization and for longitudinal epidemiological studies (12, 37). A new and unpublished collection (n=229, corresponding to *A. baumannii*, n=228; and *A. lwoffii*, n=1) was obtained between 2004 and 2008 from several hospitals and two clinical analysis laboratories, located in North and Centre of Portugal. These isolates were included for longitudinal epidemiological studies, and plasmid and carbapenem resistance characterization.

International clinical isolates from Brazil (*A. baumannii*, n=17) and Czech Republic (*A. baumannii*, n=9) were included for population structure analysis and comparison with Portuguese isolates. Concerning the Czech Republic isolates, the associated carbapenem resistance mechanism was also investigated.

The main characteristics of all isolates included in the present thesis are summarized in Table 5. Control and reference strains are indicated on Table 6.

6.1.2 Media and growth conditions

Different media were used for isolation and growth of *Acinetobacter* spp isolates. Isolates growth was performed aerobically at 37 ºC (8-24h).

- *Cystine Lactose Electrolyte Deficient* (CLED) Agar medium (Liofilchem, Italy), an electrolyte deficient and non-inhibitory differential medium, was routinely used for bacterial growth;
- *Tryptone Soya Broth* (TSB) (Oxoid Ltd.) supplemented with glycerol (15%) was used to stock *Acinetobacter* spp. cultures at -80 ºC;
- *Mueller-Hinton II* (MH) Agar, a non-selective and non-differential medium, was used for antimicrobial susceptibility testing;
- *Luria-Bertani* (LB) Broth, a nutritionally rich medium, was used to promote the growth of bacteria;

- *Blood agar plate* (BAP), a differential media generally used to isolate fastidious organisms and detect hemolytic activity, was employed to cultivate bacteria for agarose-embedded bacterial genomic DNA preparation.

6.1.3 Species identification

Isolate's identification and antimicrobial susceptibilities were firstly conducted within each hospital by the automated Vitek 2 system (bioMérieux, Marcy l'Étoile, France). Identification of the isolates was, thereafter, confirmed at our laboratory by API 20NE system (bioMérieux, Marcy l'Étoile, France) with subsequent sequencing of 16S rRNA gene and by the detection of species-specific β-lactamases: OXA-51-like for *A. baumannii* and OXA-134-like for *A. lwoffii* (18, 46, 49)

6.1.4 Antimicrobial susceptibility testing

6.1.4.1 Disc diffusion method

In vitro susceptibility for β-lactams, aminoglycosides, tetracyclines and fluoroquinolones was determined by standard disc diffusion method - the *Kirby-Bauer* method - following the CLSI (*Clinical Laboratory Standards Institute*) guidelines (10). The routine panel of antibiotics tested in *Acinetobacter* spp. included: amoxicillin (10 µg), amoxicillin–clavulanic acid (20+10 µg), piperacillin (100 µg), ceftazidime (30 µg), cefepime (30 µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), imipenem (10 µg), meropenem (10 µg), aztreonam (30 µg), gentamicin (10 µg), tobramycin (10 µg), netilmicin (30 µg), amikacin (30 µg), tetracycline (30 µg) and ciprofloxacin (5 µg) (Oxoid Ltd, Basingstoke, Hants, UK). The accuracy of the test was monitored by using the control strain *Pseudomonas aeruginosa* ATCC 27853 (susceptible and wild type strain). Inhibition zone diameters were measured and isolates were reported as susceptible (S), intermediate (I) or resistant (R) according to the CLSI Zone Diameter Interpretative Standards (10).

6.1.4.2 Agar dilution method

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation. MICs for minocycline, tigecycline and colistin were performed by an agar dilution technique (10) where MH plates are supplemented with antibiotic solutions in a range of concentrations with a two-fold dilution series. The inocula were transferred

both to the series of agar plates with an inoculum-replicating apparatus (Multipoint inoculator A400, Denley, Sussex, England), and to a control plate without antimicrobial agent. For minocycline, MICs results were interpreted according to CLSI guidelines (10). For colistin, the resistance breakpoint was defined as a MIC ≥4 µg/ml following EUCAST guidelines (http://www.eucast.org/clinical_breakpoints/). As no tigecycline interpretive criteria currently exist for *Acinetobacter* spp., the Food and Drug Administration approved breakpoints for members of the *Enterobacteriaceae* family of ≤ 2, 4, and ≥8 µg/mL (designating tigecycline susceptible, intermediate and resistant, respectively), which were applied as provisional MIC breakpoints.

6.1.4.3 *Epsilon* test (*E-test*)

The *E-test* (AB Biodisk) method combines the principle of the agar diffusion test with the determination of the MIC value. A predefined stable antimicrobial gradient is present on a thin inert carrier strip. When this *E-test* strip is applied onto an inoculated agar plate, there is an immediate release of the drug. Following incubation, an elliptical zone of inhibition is produced. The point of intersection of the inhibitory zone edge and the calibrated carrier strip indicates the MIC value over a wide concentration range (>10 dilutions). MICs for amoxicillin, amoxicillin–clavulanic acid, ticarcillin, ticarcillin–clavulanic acid, piperacillin, piperacillin–tazobactam, ceftazidime, cefotaxime, cefpirome, cefpime, cefoxitin, cefalotin, imipenem and meropenem were determined by *E-test* in representative isolates.

6.1.5 Phenotypic characterization of antibiotic resistance

6.1.5.1 Detection and characterization of carbapenemases

Carbapenemase-producing clinical isolates usually display high carbapenem MICs. In accordance, several methodologies have been proposed for the routine phenotypic detection of carbapenemase activity in clinical isolates. Although generally considered as fast, efficacious and easy-to-perform methods, some concerns regarding their reliability emerged and have been pointed out (38).

6.1.5.1.1 Modified Hodge Test (MHT)

The Modified Hodge Test is based on the capacity displayed by a carbapenemase-producing isolate to allow the growth of a carbapenem susceptible strain (*E.coli* ATCC 25922) towards a carbapenem disk, resulting in a characteristic cloverleaf-like indentation (27). A cell suspension of *E.coli* ATCC 25922 was prepared in 5 ml of

0.85% physiological saline and adjusted to McFarland 0.5 standard. This suspension was diluted to 1:10 and streaked on MH agar plates, allowing to dry 3-5 minutes. An imipenem or meropenem disk (10 µg) was placed in the center of the test area. The tested isolates were streaked in a straight line from the edge of the disk to the edge of the plate. The plates were incubated at 37ºC (18-24 hours). An MHT positive test was revealed by a clover leaf-like indentation of the *E.coli* ATCC 25922 growing along the test organism growth streak within the disk diffusion zone.

6.1.5.1.2 Imipenem - ethylenediaminetetraacetic acid (EDTA) double disk synergy test

Metallo-β-lactamases belong to Ambler class B and require zinc for their catalytic activity, being inhibited by metal chelators, such as EDTA. The disk approximation test with EDTA is often used as a screening method for metallo-β-lactamases. In the presence of a metallo-β-lactamase, appeared a synergistic inhibition zone around the β-lactam disk (imipenem, ceftazidime, and cefepime) (28). Cell suspensions from strains were adjusted to the McFarland 0.5 standard and used to inoculate MH agar plates, where an imipenem disk (10µg) and a blank filter paper disk were placed at a distance of 10 mm (edge to edge). Then, 10 µl of a 0.2 M EDTA solution was added to the blank disk. It is important to remark that most papers describe the use of 0.5 M EDTA (27, 28), however disks containing solely EDTA can produce an inhibitory action against some bacteria due to permeabilization of the outer membrane and can lead to false positive results, especially when is used at higher concentrations (38). After overnight incubation, the presence of even a small synergistic inhibition zone was interpreted as positive.

6.1.5.1.3 Detection of carbapenemase activity by a microbiological assay

Detection of carbapenemase activity was carried out using enzymatic crude extracts that were prepared with overnight cultures (37ºC, 18 hours) grown in 50 mL of TSB (Oxoid). Cells were harvested by centrifugation at 3000 rpm, for 10 minutes at 4℃, washed twice with 10 mL of 0.85% physiological saline and suspended in 500 µL of sterile distilled water. Lysis was obtained with 5-6 freeze-thaw cycles. The microbiological assay included the preparation of a cell suspension of *E. coli* ATCC 25922 in 5 mL of 0.85% physiological saline and adjusted to McFarland 0.5 standard. This suspension was streaked to the plates allowing to dry 3-5 minutes and imipenem discs (10µg) impregnated with 10 µl of each enzymatic extract alone or with 10 µl of a

0.5 M EDTA solution were added. Plates were incubated at 37ºC overnight. Inactivation of imipenem by a carbapenemase was revealed by the *E. coli* ATCC 25922 strain growth within the expected inhibition zone. If a MBL was present, the inactivation of imipenem was inhibited by the addition of EDTA.

6.1.5.1.4 Detection of carbapenemase activity by spectrophotometric assay

Investigation of carbapenemase activity in crude extracts was performed by UV spectrophotometric assays. The hydrolytic activity of crude extracts was determined against 100 µM imipenem in 100 mM phosphate buffer (pH 7.0), and measurements were carried out at 297 nm. Positive controls included VIM-2-producing *Pseudomonas aeruginosa*, IMP-5-producing *A. baumannii* and OXA-40-producing *A. baumannii* (Table 6).

6.1.5.1.5 Isoelectric focusing of carbapenemases

Isoelectric focusing (IEF) separates proteins by charge at their isoelectric points (pI) in an electrophoretically-produced pH gradient. Samples (crude extracts) were applied on the surface of the polyacrylamide gel with a pH gradient of 3-9 (PhastGel IEF 3-9, Amersham Biosciences) and a pre-focusing phase was performed in order to establish the gradient. The IEF conditions were set according to the apparatus PhastSystem (Pharmacia AB) manufacturer instructions. Detection of β-lactamase bands was facilitated by overlaying the gel with nitrocefin. The corresponding pIs were determined by comparing the migration distance between the obtained bands and those from a ladder pre-prepared with crude extracts of β-lactamase-producing strains containing known pIs (5.4, 5.7, 7, 7.4 and 8.2).

6.1.5.2 Detection of antibiotic resistance mediated by efflux systems

To determine the presence of an efflux mechanism involved in *A. baumannii* resistance to imipenem, tigecycline and amikacin, a MIC assay was conducted for the protonic efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, USA) using the agar dilution method, according to the guidelines established by CLSI (10). MH agar plates supplemented with the antibiotic under study (imipenem, tigecycline or amikacin) were prepared with and without CCCP. The final concentration of CCCP in the MH agar was 16 mg/L, corresponding to a quarter of MIC (6) previously determined. An inoculum of 0.5 Mcfarland standard of each isolate was inoculated onto

MH medium containing serial dilutions of the antibiotic. Four-fold or more reduction (41) of imipenem, tigecycline or amikacin MICs in the presence of CCCP was considered indicative for the presence of an efflux system mode of resistance against these antibiotics.

6.1.6 Molecular Biology Techniques

6.1.6.1 Extraction of bacterial DNA

The different methods used for the isolation of chromosomal or plasmid DNA included the disruption of the cell, removal of cell debris and proteins, and the selective precipitation of double-stranded DNA of high molecular weight.

6.1.6.1.1 Genomic DNA

In most cases DNA samples were extracted by a boiling method. A loopfull from a colony was suspended in 200 µL of distilled water. The suspension was boiled for 10 min and centrifuged at 13000 rpm for 15 min. The supernatant was used as a template (2µL in a final volume of 25 µL) for PCR reactions. DNA was stored at –20℃ until further use. In particular cases (preparation of DNA templates for MLST PCR reactions) DNA was extracted by a commercial kit (InstaGene™Matrix, Bio-Rad Laboratories, Inc., Hercules, CA). Briefly, an isolated bacterial colony was suspended in 1 mL of sterile water in a microfuge tube and centrifuged for 1 min at 10000–12000 rpm. The supernatant was removed and 200 µl of InstaGene matrix were added to the pellet and incubated at 56ºC for 15–30 min. The mixture was vortexed at high speed for 10 sec and the tube was placed in a 100ºC boiling water bath for 8 min. The mixture was vortexed at high speed for 10 sec and centrifuged at 10000–12000 rpm for 2–3 min. The supernatant was used as the template for PCR and the remainder was stored at -20ºC.

Standard procedures for DNA preparation do not yield intact, high molecular weight DNA molecules. Large DNA molecules are so fragile that they are sheared by mechanical forces during isolation. To prevent breakage of large DNA molecules, extraction was performed with intact cells embedded in agarose that were lysed and deproteinized *in situ* according to reference methods (9) . Briefly, individual bacterial colonies grown overnight on BAP medium, were suspended directly in 1 mL of *Cell Suspension Buffer* (100 mM Tris:100 mM EDTA, pH 8.0) with cotton swabs moistened with the same buffer. Aliquots of 200 µL of the cell suspensions were transferred to 1.5-

mL microcentrifuge tubes. Proteinase K (20 mg/mL stock solution) was added (10 µL) to each tube and mixed gently with pipet tip. Seakem Gold agarose (Lonza, Rockland, ME USA) was prepared in *TE Buffer* (10 mM Tris:1 mM EDTA, pH 8.0) and 20% SDS (pre-heated to 55ºC) to a final concentration of 1.0% agarose:1.0% SDS (sodium dodecyl sulfate), and maintained at 55℃ in a shaki ng water bath. An equal volume of agarose (200 µL) was mixed with each bacterial suspension with the help of a pipette. This bacterium-agarose mixture was immediately added to plug molds (Bio-Rad Laboratories). The plugs were allowed to solidify for 15 min at room temperature and then transferred to 50 mL polypropylene screw-cap tubes containing 5 mL of *Cell Lysis Buffer* (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) and Proteinase K (final concentration of 0.1 mg/mL in the lysis buffer). They were incubated in a shaking water bath at 55℃ for 2 h. After the completion of prote olysis, the plugs were transferred to 50-mL tubes containing 8 to 10 mL of sterile, preheated (50℃) distilled water and incubated for 10 min at 50℃ with gentle mixing in a shaker water bath. Subsequently, four 50℃ washes were done in a shaking water bath for 15 min each with 8 to 10 ml of preheated (50℃) *TE buffer*. The plugs were then cooled to room temperature in *TE buffer*. At this point, they could be used immediately or stored at 4℃ in 1 mL of *TE buffer*.

6.1.6.1.2 Plasmid DNA

Plasmid DNA was obtained using a commercial kit and following the manufacturer´s instructions (QIAGEN Plasmid Purification Midi Kit). Briefly, the protocol was based on a modified alkaline lysis procedure followed by binding of plasmid DNA to an anion-exchange resin and further elution with an appropriate buffer. After this step the plasmid DNA was concentrated and desalted by isopropanol precipitation.

6.1.6.1.3 DNA digestion

Comparison of restriction fragment length polymorphism (RFLP) patterns was used to analyze similarities among plasmids. The restriction enzymes (*Eco*RI, *Bam*HI and *Hind*III) were selected based on the plasmid nucleotide sequences deposited on GenBank for bla_{OXA-40}-carrying plasmids. Reaction conditions were implemented according with the manufacturer's instructions. The incubation time was overnight (16-24h) at the optimal temperature for each enzyme (25 ºC or 37 ºC).

Genomic DNA embedded in agarose plugs was digested in the same way to liquid-phase reactions, but providing higher enzyme concentrations and longer incubation

times. One slice of 2 mm-thick from each plug was pre-equilibrated in the appropriate restriction enzyme buffer for 10-30 minutes and then digested [1X restriction enzyme buffer, 10-30 U of enzyme (*Apa*I, I-*Ceu*I, S1) and 1X BSA)] at 25 ºC or 37 ºC and appropriated incubation time (15 min-16h).

6.1.6.2 Extraction of bacterial messenger RNA (mRNA)

The relative quantification of mRNA by quantitative real-time-PCR (qRT-PCR) can reflect the gene-expression profile of a given organism. However, RNA is relatively unstable, being very sensitive to degradation which occurs through cleavage with ribonucleases (RNases). For an accurate quantification of mRNA levels with quantitative real-time-PCR it is necessary to obtain intact mRNA. In this study RNA templates were prepared using an RNeasy kit (QIAGEN Sciences, MD) according to the manufacturer's instructions. This commercial kit combines the technology of the selective binding properties of a silica-based membrane with the speed of microspin technology. Briefly, bacterial cells were grown aerobically in LB broth until mid-log phase. Then, cells were lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol was added to provide appropriate binding conditions, and the sample was applied to an RNeasy Mini spin column, where the total RNA was attached to the membrane and contaminants were washed away. RNA was eluted in 30µl water treated with 0.1% diethyl pyrocarbonate (DEPC). DEPC is a strong, but not absolute, inhibitor of RNases that works by covalently modifying RNases. Residual DNA was removed by DNase I digestion following RNA isolation, using the QIAGEN RNase-Free DNase Set (QIAGEN Sciences, MD). The concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}).

6.1.6.3 Amplification of Nucleic Acids

6.1.6.3.1 Polymerase Chain Reaction - general conditions

In this study, PCR reactions, both by simplex and multiplex schemes, were performed with the purpose of detection, identification and characterization of several DNA sequences.

Primers, reagents and amplification conditions are described on Table 7. PCR amplifications were performed in termocyclers *iCycler* (BioRad) or *MyCycler* (BioRad). Primers synthesis was carried out by STAB Vida, Portugal.

PCR products purification was performed before sequencing or labeling. This purification was achieved by using a commercial kit, the *Wizard SV Gel and PCR CleanUp System* (Promega), following the manufacturer's instructions. Briefly, after DNA binding to a silica membrane, residual primers, excess of nucleotides and *Taq* polymerase were removed by washing with an ethanol-based solution. Finally, the DNA was eluted in nuclease-free water.

The amplified fragments were sequenced by STAB Vida, Portugal (Taq DyeDeoxy Terminator Cycle Sequencing and Model 373A gel apparatus, Applied Biosystems). Sequence analysis was conducted using the free sequence analysis software for PCs *Chromas Lite* (Technelysium Pty Ltd). Sequences were further compared with others, using the Basic Local Alignment Search Tool (BLASTN) from the public database of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ClustalW2 program was used to align multiple DNA sequences and to organize them according to their identities (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

6.1.6.3.2 Quantitative Real Time PCR (qRT-PCR)

Real-time PCR is PCR-based technique which is used to amplify and simultaneously evaluate in real-time how the reaction proceeds. A DNA-binding dye binds to all double-stranded (ds)DNA in PCR, causing dye fluorescence. An increase in DNA product during PCR leads therefore to an increase in fluorescence intensity, which is measured at each cycle, thus allowing DNA concentrations to be quantified. Thus, quantitation is the most common use for real-time PCR, often referred to as quantitative PCR or qPCR [Real-Time (qRT-PCR)].

6.1.6.3.2.1 Efflux system AdeABC - Gene expression

AdeABC is an efflux pump that belongs to the resistance-nodulation-cell division (RND) family and displays a three-component structure: AdeB integrates the *trans*-membrane component, AdeA forms the inner membrane fusion protein, and AdeC forms the outer membrane protein. (31), (35) (32) The expression of *AdeABC* is regulated by a two-component system that includes a response regulator (AdeR) and a sensor kinase (AdeS), whose genes are located upstream AdeABC locus and are transcribed in the opposite direction. Point mutations in *adeS* and *adeR* have been associated with overexpression of AdeABC and consequently with multidrug resistance.

To investigate whether the AdeABC efflux pump plays a role in decreased susceptibility to amikacin and tigecycline, the expression of *adeB* was measured by qRT-PCR.

Oligonucleotide primers for the *adeB* and 16S rDNA genes were described elsewhere (24, 35) (25) (Table 7). 16S rRNA gene was used as a housekeeping gene to normalize levels of *adeB* transcripts. *A. baumannii* ATCC 19606 was used as a control strain. Negative-control reactions included equal concentrations of RNA and all reagents except reverse transcriptase. Reverse transcription was performed on an iCycler iQ5™ Real-Time PCR Detection System (BioRad) using an iScript™ SYBR® Green RT-PCR Kit (Bio-Rad) at a 1X concentration containing 125 nM of each primer and a 1:100 final dilution of the cDNA product. Following PCR cycling, melting point data were collected and a dissociation curve was examined for each well. Each sample was run in triplicate. The ΔCT for *adeB* was calculated comparatively to that for the 16S rRNA housekeeping gene, and the ΔΔCT was calculated relatively to *A. baumannii* ATCC 19606, which was used as a calibrator sample.

6.1.6.3.3 Nucleic acids separation and visualization

The detection and characterization of DNA and RNA was performed by agarose gel electrophoresis. Agarose gel electrophoresis runnings were performed using either continuous or pulsed-field systems.

6.1.6.3.3.1 Electric continuous-field gel electrophoresis

PCR products and mRNA were resolved by conventional agarose gel electrophoresis in an electric continuous-field. Samples were loaded in agarose 1.5% w/v gels in TAE buffer (40 mM Tris base, 20mM Acetic acid, 1 mM EDTA, pH 8.3) and fragments were separated at 90V for 45 minutes. Plasmid DNA was resolved in agarose 0.8% w/v gels in 0.5X TBE buffer at 90V for 180 min. Gels were stained with ethidium bromide (0.5µg/mL) and the DNA was visualized by UV light trans-illumination at 302 nm. The specific size of the linear product(s) was determined by the routine inclusion of a DNA standard containing a range of fragments of known sizes. The used DNA standards were *100 bp DNA Ladder* (Promega) or *250 bp DNA Ladder* (Invitrogen). The specific size of plasmidic band(s) was determined by the inclusion of plasmid DNA from strains *E.coli* V517 (that contains eight plasmids with a range of sizes of 2.1-54.4 Kb) and *E. coli* NTCT 50192 (that presents four plasmids of 7.0, 36.2, 63.8 and 148.5 Kb).

6.1.6.3.3.2 Electric Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed field gel electrophoresis (PFGE) lies on an electric field that periodically changes direction to a gel matrix allowing the separation of large deoxyribonucleic acid (DNA) molecules. Electrophoresis of the prepared samples was performed on the contour-clamped homogeneous electric field (CHEF) mapper system (Bio-Rad Laboratories, Richmond, CA) by using 1.6% w/v pulsed-field-certified agarose (Seakem Gold agarose, Lonza, Rockland, ME USA) with 2 liters of standard 0.5X TBE (65 mM Tris, 22.5 mM boric acid, 1.25 mM EDTA) running buffer. The electrophoretic conditions used were as follows: i) *Apa*I macrorestriction - initial switch time, 5 s; final switch time, 13 s; run time, 25 h; ii) S1 and I-*Ceu*I macrorestriction - initial switch time, 5 s; final switch time, 60 s; running time, 20 h ; angle, 120°; gradient, 6.0 V/cm; temperature, 14°C; ramping factor, linear (12). After electrophoresis, gels were stained for 30 min in 1 liter of sterile distilled water containing 100 mL of ethidium bromide (10 mg/mL) and destained with two washes in 1 liter of distilled water (of 30 min each).

6.1.6.4 DNA transfer and hybridization

6.1.6.4.1 DNA separation

In order to determine whether β-lactamase genes were chromosomal or plasmid located the *I-Ceu*I endonuclease technique (New England Biolabs, Beverly, MA) was conducted. This enzyme digests a 26-bp sequence in *rrn* genes for the 23S large-subunit rRNA. Whole-cell DNA was digested with 4 units of *I-Ceu*I and the resulting fragments (usually seven, corresponding to the number of copies of the ribosomal RNA gene present in each cell) were separated by PFGE. Electrophoresis was performed using 0.9% w/v pulsed-field-certified agarose (Seakem Gold agarose, Lonza, Rockland, ME USA) and 0.5X TBE (65 mM Tris, 22.5 mM boric acid, 1.25 mM EDTA) as the running buffer. The electrophoretic conditions were described above (section 6.1.6.3.3.1). After electrophoresis, gels were stained for 30 min in 400 mL of sterile distilled water containing 40 mL of ethidium bromide (10 mg/mL).The sizes of the obtained fragments were determined by comparison with those from *E.coli* K12.

S1 nuclease technique was used for plasmid size determination and replicase genes identification. With this restriction analysis, plasmids were converted into unit-length linear molecules, which migrate at rates that allow, by comparison with linear markers, an accurate sizing of the bands (2). The digested products were separated by PFGE according to previous described running conditions (section 6.1.6.3.3.1).

6.1.6.4.2 DNA transfer and fixation

Southern Blot methodology includes an overnight transfer of DNA to a positively charged nylon membrane using an upward-transfer method. For this purpose, the gel was irradiated for 1min and 30s on the transilluminator. Then, it was denatured by soaking it in 500 mL of an alkaline solution of 0.4N NaOH for 90 min. This denaturation step of the DNA into single strands allows the hybridization with the probe. The transfer was setup from bottom to top by using (1) a large dish filled with 0.4N NaOH with glass plate on top of it to rest the gel, (2) one piece of Whatman paper cut to the width of the gel and length such that the wick is in contact with the bottom of the dish and pre-wet in 0.4N NaOH, (3) the agarose gel, turned upside down because the flatter side makes a better contact surface for the nylon membrane, (4) the positively charged nylon membrane Hybond N+ (GE Healthcare Life Sciences) with the exact size of the gel, with a nick in the corner for orientation and pre-wet in 0.4N NaOH, (5) two pieces of blotting paper cut to size of the gel, (6) paper towels on top to pull the NaOH through the gel and (7) an additional weight was added to keep blot in place. The entire gel was covered with plastic wrap. The transfer occurred for 48 h. After this time the membrane was removed from the gel and washed in 0.5M Tris, pH 7-8 / 2X SSC for 5 min. Then the membrane was cross-linked with UV light. The resulting membrane was immediately used for hybridization or was stored at -20ºC.

6.1.6.4.3 DNA labeling and hybridization

The DNA probes included *bla*$_{OXA-40}$, 16S rRNA and replicase genes, amplified by PCR (Table 7). The labeling was obtained with the probe denaturation by boiling and mixing with the labeling buffer and the alkaline phosphatase enzyme. Then formaldehyde was added to covalently cross-link the enzyme to the probe and the mixture was incubated at 37ºC for 30 min. During the labeling process of the probe the membrane was pre-hybridized with the AlkPhos Direct™ hybridization buffer (GE Healthcare Life Sciences) for 15 min at 55 ºC. After that, the probe was added to the hybridization buffer and hybridization was performed overnight at 55ºC.

6.1.6.4.4 Post hybridization washes, signal generation and detection

After completion of hybridization, stringency washes were performed to remove residual labeled probe. Hybridized blots were detected by chemiluminescence with CDP-Star™ (GE Healthcare Life Sciences). After 2 min of incubation, the membrane was exposed to Hyperfilm™ ECL™ (GE Healthcare Life Sciences) for 2 h.

6.1.6.4.5 Stripping

Following exposure of a Southern Blot, it might be needed to strip off the probe, while maintaining the nucleic acid target on the membrane, in order to hybridize the DNA with another probe. DNA probes were eliminated by washing the nylon membrane with a denaturing and boiling solution (SDS at 1%) for 1-2h, followed by a brief wash with 2X SSC solution at room temperature.

6.1.7 Typing methods

The sources and pathway(s) of transmission for epidemic strains can be determined using typing methods, however it is not well established which one is more reliable for typing *Acinetobacter* spp. In this study, several typing methods were used in order to characterize the population structure of carbapenem-resistant *A. baumannii* in Portugal, Czech Republic and Brazil.

6.1.7.1 DNA macrorestriction analysis of *Apa*I digests by pulsed-field gel electrophoresis

DNA macrorestriction followed by PFGE has been considered the gold standard method for typing *A. baumannii* clinical isolate (3). Generally, *Apa*I is used for restriction of intact chromosomal DNA and the resulting chromosomal fragments are separated by electrophoresis. The genomic DNA patterns obtained with this electrophoresis are highly specific for different strains and the resulting fingerprint profiles can be compared visually or using specialized computer programs. However, it is a laborious method that requires several days before generating a result, is considered more suitable for short-term outbreak investigations and is generally restricted to analyze small sets of isolates (1). In addition, the comparison process of results obtained from different laboratories remains a difficult task.

Strain relatedness among *A. baumannii* isolates was determined according to Tenover's criteria (43), where isolates are considered as i) **indistinguishable** (if their restriction patterns have the same numbers of bands and the corresponding bands are the same apparent size, with the isolates being considered to represent the same strain); ii) **closely related** (if PFGE patterns differ by changes consistent with a single genetic event, which typically results in two to three different bands); iii) **possibly related** (if PFGE patterns differ by changes consistent with two independent genetic events i.e., four to six band differences) and iv) **unrelated** (if PFGE patterns differ by

changes consistent with three or more independent genetic events, generally seven or more band differences). In addition, PFGE patterns were analyzed by InfoQuestTM FP software version 5.4 (BioRad Laboratories) with Dice coefficient analysis of peak positions executed. The unweighted-pair group method using average linkages (UPGMA) was applied, and the bandwidth tolerance was set at 1.5%. Isolates clustering together with a >85% level of similarity were considered to belong to the same PFGE type (39, 42).

6.1.7.2 PCR-based sequence group (SG) identification

This PCR-based typing method relies on two multiplex PCRs, used for identification of allele variations in the *ompA* (outer-membrane protein A), *csuE* (part of a pilus assembly system required for biofilm formation) and *bla*$_{\text{OXA-51-like}}$ (the intrinsic carbapenemase gene in *A. baumannii*) genes. The combination of different alleles allows to assign the isolates onto sequence groups which seems to correlate with the major *A. baumannii* epidemic lineages: i) SG1 isolates correspond to European Clone II; ii) SG2 isolates correspond to European Clone I; iii) SG3 is an outbreak strain with a lower expression (16, 44, 45). After the description of these three main groups, Higgins *et al* (2010) (22) performed a larger study that included outbreak strains from all over the world, increasing the number of sequence groups for eight and renaming them as WW1-WW8 (WW standing for worldwide). The primers and PCR conditions for this typing method are listed on Table 7 (45).

6.1.7.3 Sequence-based typing of *bla*$_{\text{OXA-51-like}}$ genes

The OXA-51 class D β-lactamase was first identified in *A. baumannii* in 2004 and since then it has been suggested that *bla*$_{\text{OXA-51-like}}$ genes are ubiquitous in *A. baumannii (16)*. Minor variations within the sequence encoding OXA-51 have subsequently been reported, constituting the OXA-51-like subgroup of enzymes (15, 16). With the increasing number of isolates being typed with the SG identification method, it was raised the possibility that isolates containing closely related OXA-51-like enzymes would be closely related and the sequencing of *bla*$_{\text{OXA-51}}$ variant could constitute itself a typing scheme (16, 19). The sequence typing of *bla*$_{\text{OXA-51-like}}$ genes was performed for representative isolates of each PFGE type. The primers and PCR conditions are listed on Table 7. Sequence analysis was performed at the National Center for Biotechnology Information (NCBI) web site using the Basic Local Alignment Search Tool (BLAST) software and the nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

6.1.7.4 Multilocus Sequence typing (MLST)

MLST is used for characterizing bacterial isolates via the *internet* and has been applied successfully for the epidemiologic characterization of a variety of clinically important bacterial pathogens (3). The method relies on the comparison of nucleotide sequence data from internal fragments (approximately 450- to 500-bp) of protein encoding housekeeping genes. These genes codify for products that play vital functions, being present in all isolates of a given species, and mutations within them are assumed to be neutral (3). For each gene fragment, the different sequences are assigned as distinct alleles, and each isolate is defined by the alleles at each of the housekeeping loci (the allelic profile or sequence type [ST]) (3).

6.1.7.4.1 MLST scheme develloped by Bartual *et al* (2005)

This MLST scheme, hosted by the Oxford database (http://pubmlst.org/abaumannii/), is based on the allelic variations in seven housekeeping genes: citrate synthase (*gltA*), DNA gyrase subunit B (*gyrB*), glucose dehydrogenase B (*gdhB*), homologous recombination factor (*recA*), 60-kDa chaperonin (*cpn60*), glucose-6-phosphate isomerase (*gpi*), and RNA polymerase σ^{70} factor (*rpoD*) (3). Specific primers and amplification conditions are listed on Table 7. For the internal fragments of *gpi* and *gyrB* loci the PCR reaction mixture included BSA and DMSO at final concentrations of 0.4% and 10% respectively. Additionally to the primers described in the database, another group of primers for the same loci, was applied (Table 7) with the advantage of being used both to amplify and sequencing the seven loci and the preparation of DNA templates did not included the use of InstanGene Matrix (Bio-Rad Laboratories, Hercules, CA).

6.1.7.4.2 MLST scheme developed by the Institut Pasteur

The MLST scheme developed by the Institut Pasteur (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html) for *A. baumannii* (and closely related species) uses internal fragments of the following seven housekeeping genes: 60-KDa chaperonin (*cpn60*), elongation factor EF-G (*fusA*), citrate synthase (*gltA*), CTP synthase (*pyrG*), homologous recombination factor (*recA*), 50S ribosomal protein L2 (*rplB*), and RNA polymerase subunit B (*rpoB*). (34) Specific primers and amplification conditions are listed in Table 7. For this MLST scheme the DNA samples were also prepared using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA).

6.1.7.4.3 Sequences analysis and E-burst (*B*ased *U*pon *R*elated *S*equences *T*ypes)

The resulting sequences of the seven housekeeping genes amplification were analyzed using the online BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and the Pairwise Sequence Alignment tool from the *European Bioinformatics Institute* (*EBI*) (http://www.ebi.ac.uk/Tools/). Allele and ST assignments were performed on the respective *A. baumannii* MLST webpages (http://pubmlst.org/abaumannii/; http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html).

The E-Burst analysis allows grouping the isolates according to their allelic profiles and it is based on the number of Single Locus Variants (SLV), Double Locus Variants (DLV) and Satellites (SAT) for each sequence type (ST). In some cases it is also possible to identify the potential Ancestral Type (AT). This analysis was performed using the eBURST software program available at the eBurstv3 website (http://eburst.mlst.net/) (17).

6.1.8 Transformation assays

Genetic transformation occurs when a host organism takes up foreign DNA and expresses the foreign gene. Electroporation is a physical process that transiently permeabilizes cell membranes with an electrical pulse, thus permitting cell uptake of a wide variety of biological molecules, including plasmid DNA (14). Transformation was performed using *A. baylyi* ADP1 as the recipient strain (Table 6). MicroPulser Electroporation Apparatus (Bio-Rad) was used according to manufacturer's instructions, following the high efficiency electrotransformation of *E. coli* protocol.

6.1.8.1 Electrocompetent cells preparation

The *A. baylyi* ADP1 electrocompetent cells were prepared according to the manufacturer's instructions. Briefly, 1/100 volume of a fresh overnight culture was inoculated in 500 mL of LB medium. This suspension was incubated at 37ºC and shaking at 150 rpm to an OD_{600} of approximately 0.5-0.7. The cells were chilled on ice for 20 min and maintained as close to 0ºC as possible in all the subsequent steps. The cells were harvested at 4000 g for 15 min at 4ºC. The supernatant was discarded and the pellet resuspended in 500 mL of ice-cold 10% glycerol. The cells were harvested again at 4000 g for 15 min at 4ºC. After discarding the supernatant, the pellet was resuspended in 250 mL of ice-cold 10% glycerol. The harvest procedure was repeated

followed by pellet resuspension in 20 mL of ice-cold 10% glycerol. Finally, a last centrifugation was performed in the same previous conditions and the cell pellet was resuspended in a final volume of 1-2 mL of ice-cold 10% glycerol. This suspension was frozen in aliquots in dry ice and stored at -70ºC, being stable under these conditions for at least 6 months.

6.1.8.2 Electroporation protocol and transformants selection

For the electroporation process bacterial competent cells were thaw on ice. In a cold 1.5-mL microcentrifuge tube 40 µL of the cell suspension were mixed with 5 µL of plasmid DNA (that had been eluted in a low ionic strength buffer – TE). The mixture was incubated on ice for 1 min. The mixture of cells and DNA was transferred to a cold electroporation cuvette and submitted to one pulse with a voltage of 3.0 kV. Then, 1 mL of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to the cuvette. The cell suspension was transferred to a 15 mL polypropylene tube and incubated at 37ºC for 1 h, shaking at 225 rpm. After incubation, the suspension (200 µL) was plated on TSA supplemented with imipenem (0.5 and 1 mg/L).

Table 5 – Characteristics of *Acinetobacter* spp. isolates included in this thesis.

Country	Origin	Period	Species (n)	Sources (n)	Applicability	Reference(s)
Portugal	HPA	1995[a]	*A. baumannii* (1)	sputum (1)	Epidemiological studies	This study, (12)
	HUC	1998-2000[a]	*A. baumannii* (2)	exsudate (1); urine (1)	Epidemiological studies; control strains for PCR reactions	This study, (12)
		2001-2003[a]	*A. baumannii* (5)	bronchial secretion (4); sputum (1)	Epidemiological studies; plasmids characterization;	This study, (12) (37)
			A. haemolyticus (2)	pus (1); urine (1)		This study
		2004	*A. baumannii* (30)	pus (3); bronchial secretion (6); catheter (2); blood (4); urine (12); sputum (3)	Epidemiological studies; plasmids characterization; carbapenem resistance mechanisms	
	HGSA	2006	*A. baumannii* (28)	pus (1); bronchial secretion (13); catheter (6); urine (6); other (2)		
		2008	*A. baumannii* (105)	pus (7); bronchial secretion (40); catheter (2); urine (34); wound (5); surgical site (5); blood (4); CRL[c] (1); intraeritoneal fluid (4); ascitic fluid (2); other (1)		
			A. lwoffii (1)	incubator humidity chamber (1)		
	HPH	2006-2007	*A. baumannii* (21)	NA[d]		This study
	HST	2006-2008	*A. baumannii* (16)	intraeritoneal fluid (3); pus (3); urine (2); catheter (1); bronchial secretion (1); other (5)		
	CHCB	2007	*A. baumannii* (5)	sputum (2); other (3)	Epidemiological studies; carbapenem resistance mechanisms	
	HA	2007	*A. baumannii* (21)	NA[c]		
	Clinical laboratories[b]	2007	*A. baumannii* (2)	wound (1); urine (1)		

Table 5 – Characteristics of *Acinetobacter* spp. isolates included in this thesis (cont.)

Country	Origin	Period	Species (n)	Sources (n)	Applicability	Reference(s)
Brazil	HV	2006	*A. baumannii* (1)	tracheal secretions (1)	Epidemiological studies	This study, (8)
	HGB	2007	*A. baumannii* (3)	anal swab (1); tracheal secretions (2)		
	HFL	NA	*A. baumannii* (1)	wound (1)		
	HQD	2007	*A. baumannii* (4)	necrotizing fasciitis (1); urine (1); bronchoalveolar lavage (2)		
	HCD	2007	*A. baumannii* (5)	blood (1); tracheal secretions (1); abdominal secretion (1); surgical site (1)		
	HBD	2006-2007	*A. baumannii* (2)	bronchoalveolar lavage (2)		
	HUAP	2007	*A. baumannii* (1)	blood (1)		
Czech Republic	UHP	2008	*A. baumannii* (9)	Sputum (4), wound (2); urine (1); bronchoalveolar lavage (1); catheter (1)	Epidemiological studies; carbapenem resistance determinants	This study

[a] Indicates representative isolates selected from a previously published collection, included for a longitudinal analysis of carbapenem-resistant *A. baumannii* population and for plasmidic characterization; [b] Isolates from outpatients; [c] CLR, cephaloraquidian liquid; [d] NA, information not available

HPA, Hospital Padre Américo (Penafiel), HUC, Hospitais Universitários de Coimbra (Coimbra), HGSA, Hospital Geral de Santo António (Porto); HPH, Hospital de Pedro Hispano (Matosinhos); Hospital de São Teotónio (Viseu); CHCB, Centro Hospitalar da Cova da Beira (Covilhã), HV, Hospital Vital (Rio de Janeiro), HGB, Hospital Geral de Bonsucesso (Rio de Janeiro); HFL, Hospital Federal da Lagoa (Rio de Janeiro); HQD, Hospital Quinta D'Or (Rio de Janeiro); HCD, Hospital Copa D'Or (Rio de Janeiro); Hospital Barra D'Or (Rio de Janeiro); HUAP, Hospital Universitário António Pedro (Rio de Janeiro); University Hospital of Pilsen (Pilsen).

Table 6. Control and recipient strains used in this thesis

Strain	Source	Control/Applicability	Reference
***Acinetobacter baumannii* 65 FFC**	Laboratory bacterial collection	*bla*$_{IMP-5}$; In76; Tn*402*-like transposon	(13)
***Acinetobacter baumannii* CLA I**	Kindly provided by Dr L. Poirel	*bla*$_{OXA-40}$	(21)
***Acinetobacter baumannii* Ab13**	Kindly provided by Dr L. Poirel	*bla*$_{OXA-23}$, Tn*2006*	(11)
***Acinetobacter baumannii* Ab14**	Kindly provided by Dr L. Poirel	*bla*$_{OXA-23}$, Tn*2007*	(11)
***Acinetobacter baumannii* MAD**	Kindly provided by Dr L. Poirel	*bla*$_{OXA-58}$, IS*Aba3*-like	(36)
***Acinetobacter baumannii* ATCC 19606**	Laboratory bacterial collection	Transformation recipient strain; qRT-PCR	
***Acinetobacter baylyi* ADP1**	Kindly provided by Dr G. Bou	Transformation recipient strain	(48)
***Escherichia coli* ATCC 25922**	Laboratory bacterial collection	Carbapenemase detection control strain	(10)
***Escherichia coli* K12 BM21**	Laboratory bacterial collection	Conjugation recipient strain	(20)
***Escherichia coli* V517**	Laboratory bacterial collection	Plasmid sizing	(30)
***Escherichia coli* NCTC 50192**	Laboratory bacterial collection	Plasmid sizing	
Pseudomonas aeruginosa ATCC 27853	Laboratory bacterial collection	Antimicrobial susceptibility testing control strain	

Table 7. Oligonucleotides and PCR conditions used for the characterization of *Acinetobacter* isolates

Primer	Oligonucleotide sequence (5´to 3´)	Description	Amplicon size (bp)	PCR conditions	Reference
Identification of bacterial species					
SEQ A **SEQ B**	AGAGTTTGATCHTGGYTYAGA ACGYTACCTTGTTACGACTTC	16S rRNA gene	1500	94ºC-10min (1 cycle); 94ºC-1min, 55ºC-1min, 72ºC-1min (35 cycles); 72ºC-10min (1 cycle)	(21)
Carbapenem-hydrolyzing class D β-lactamases (CHDLs)					
OXA-23-F **OXA-23-R**	GATCGGATTGGAGAACCAGA ATTTCTGACCGCATTTCCAT	*bla*$_{OXA-23}$ gene internal fragment	501		(49)
OXA-24-F **OXA-24-R**	GGTTAGTTGGCCCCCTTAAA AGTTGAGCGAAAAGGGGATT	*bla*$_{OXA-24/40}$ gene internal fragment	246	*Multiplex PCR* 94ºC-5min (1 cycle); 94ºC-25s, 52ºC-40s, 72ºC-50s (30 cycles); 72ºC-6min (1 cycle)	
OXA-58-F **OXA-58-R**	AAGTATTGGGGCTTGTGCTG CCCCTCTGCGCTCTACATAC	*bla*$_{OXA-58}$ gene internal fragment	599		(23) This study
OXA-143-F **OXA-143-F**	TGGCACTTTCAGCAGTTCCT TAATCTTGAGGGGGCCAACC	*bla*$_{OXA-143}$ gene internal fragment	149		(5)
OXA-23C **OXA-23D**	ATGAATAAATATTTTACTTGC TTAAATAATATTCAGCTGTTT	*bla*$_{OXA-23}$ complete gene	820		(36)
OXA-FP2 **OXA-RP1**	TTCCCCTAACATGAATTTGT GTACTAATCAAAGTTGTGAA	*bla*$_{OXA-24/40}$ complete gene	980	94ºC-5min (1 cycle); 94ºC-1min, 52ºC-1min, 72ºC-2min (30 cycles); 72ºC-8min (1 cycle)	
OXA-58A **OXA-58B**	CGATCAGAATGTTCAAGCGC ACGATTCTCCCCTCTGCGC	*bla*$_{OXA-58}$ complete gene	840		

Table 7 (cont.)

Naturally occurring CHDLs					
OXA-51-F	TAATGCTTTGATCGGCCTTG	*bla*$_{OXA-51-like}$ gene internal fragment – intrinsic of *A. baumannii* species	353	94ºC-5min (1 cycle); 94ºC-25s, 52ºC-40s, 72ºC-50s (30 cycles); 72ºC-6min (1 cycle)	(49)
OXA-51-F	TGGATTGCACTTCATCTTGG				(18)
OXA-134F	ACTCAATCSACYCAAGCCA	*bla*$_{OXA-134-like}$ gene internal fragment – intrinsic of *A. lwoffii* species	223	94ºC-3min (1 cycle); 94ºC-45s, 50ºC-45s, 72ºC-1 min (35 cycles); 72ºC-5min (1 cycle)	
OXA-134_307R2	GTTTCTTGCCATCCCATTTA				
Detection of metallo-β-lactamases					
IMP-F	CTACCGCAGAGTCTTTG	*bla*$_{IMP}$ genes	587	*Multiplex PCR* 94ºC-2min (1 cycle); 94ºC-1min, 55ºC-1min, 72ºC-3min (35 cycles); 72ºC-7min (1 cycle)	(40) (7)
IMP-R	AACCAGTTTTGCCTTACCAT				
VIP-1	ACTCACCCCCATGGAGTTTT	*bla*$_{VIM}$ genes	815		
VIP-2	ACGACTGAGCGATTTGTGTG				
CHDLs vicinity – Insertion Sequences					
ISAba1A	GTGCTTTGCGCTCATCATG	tnpA of ISAba1	Variable	95ºC-5min (1 cycle); 95ºC-45s 54ºC-45s 72ºC-3min (35 cycles); 72ºC-5min (1 cycle)	(36)
ISAba1B	CATGTAAACCAATGCTCACC				
ISAba2A	AATCCGAGATAGAGCGGTTC	tnpA of ISAba2			
ISAba2B	TGACACATAACCTAGTGCAC				
ISAba3A	CAATCAAATGTCCAACCTGC	tnpA of ISAba3			
ISAba3C	AGCAATATCTCGTATACCGC	tnpA of ISAba3-like and ISAba3			
Class 1 integrons					
5´CS	GGCATCCAAGCAGCAAG	Class I Integrons	Variable,	94ºC-5min (1 cycle); 94ºC-1min, 53ºC-1min, 72ºC-6min (35 cycles); 72ºC-16min (1 cycle)	(29)
3´CS	AAGCAGACTTGACCTGA				
INT-F	GCCACTGCGCCGTTACCACC	Gene *intI*1			(26)
INT-R	GGCCGAGCAGATCCTGCACG				

Table 7– cont.

SUL1-F **SUL1-R**	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	Gene *sul1*	according with integron content	94ºC-5min (1 cycle); 94ºC-30s, 69ºC-30s, 72ºC-1min (35 cycles); 72ºC-8min (1 cycle)	(26)
ORF5-R	AGTTCTAGGCGTTCTGCG	*orf5*			(47)
MITE_Fw_2 **MITE-Rv_2**	GATAACCAATCCATTTATGACA TGACTGACCATTAAAGTCTCAA	miniature inverted-repeat transposable elements		94ºC-5min (1 cycle); 94ºC-1min, 53ºC-1min, 72ºC-6min (35 cycles); 72ºC-16min (1 cycle)	This study
Characterization of gene encoding the heat-modifiable 29-kDa outer membrane protein (*carO*)					
carO_F **carO_R**	CCATGGCTGACGAWGCAGTCGTACATGA CCATGGCAAAAGTATTAAAAGTTTTAGCAGT	Amplification and sequencing of *carO* gene	700	94ºC-3min (1 cycle); 94ºC-1min, 55ºC-1 min, 72ºC-1min (30 cycles); 72ºC-10min (1 cycle)	(33)
***bla*$_{OXA-40}$ carrying plasmids sequencing**					
OXA-40F	CGCTCAACTTAGAAATGAAAG	Primer walking and PCR mapping of *bla*$_{OXA-40}$ carrying plasmids	Variable		This study
OXA-40R	CCCTGTGTTTGAGCTTCATC				
OXA_F2	TGTTGCAGCTAGATCATCG				
OXA_R2	TGATCAGCTAGATCTATCAG				
P92_F	TCCCCTAGAGTTGTCGAACG				
P92_R	TATCACGCTCCCAGTGAT				
OXAi_40F	CTCTAGGCCAGTCCGTCTTG				
P63_R	TTATAGAAGCTTCCTATCA				
OXABa_F2	AATTGTCCATCACTGGGAG				
repA_F	TATTCCGAAGAGCCAGCCTA				
repA_R	CGAGAAAGTTGATTGCCAAA				

Table 7 (cont.)

PCR-based replicon typing of resistance plasmids in *Acinetobacter baumannii*					
gr1FW	CATAGAAATACAGCCTATAAAG	replicase p1ABSDF001	330		(4)
gr1RV	TTCTTCTAGCTCTACCAAAAT				
gr2FW	AGTAGAACAACGTTTAATTTTATTGGC	replicase Aci1 and Aci2	851		
gr2RV	CCACTTTTTTTAGGTATGGGTATAG				
gr3FW	TAATTAATGCCAGTTATAACCTTG	replicase Aci3	505		
gr3RV	GTATCGAGTACACCTATTTTTTGT				
gr4FW	GTCCATGCTGAGAGCTATGT	replicase Aci4	508		
gr4RV	TACGTCCCTTTTTATGTTGC				
gr5FW	AGAATGGGGAACTTTAAAGA	replicase Aci5	220	94ºC-7min (1 cycle); 95ºC-30s, 52ºC-30s, 72ºC-1.5min (35 cycles);	
gr5RV	GACGCTGGGCATCTGTTAAC				
gr6FW	AGCAAGTACGTGGGACTAAT	replicase Aci6	662	72ºC-5min (1 cycle)	
gr6RV	AAGCAATGAAACAGGCTAAT				
gr7FW	GAACAGTTTAGTTGTGAAAG	replicase p3ABSDF002	885		
gr7RV	TCTCTAAATTTTTCAGGCTC				
gr8FW	AATTAATCGTAAAGGATAATGC	replicases Aci8 and repM (Aci9)	233		
gr8RV	GACATAGCGATCAAATAAGC				
gr9FW	GCAAGTTATACATTAAGCCT	replicase p3ABSDF0009	191		
gr9RV	AAAAATAAACGCTCTGATGC				

Table 7 (cont.)

gr10FW	TTTCACTAGCTACCAACTAA	replicase AciX	371	
gr10RV	ACACGTTGGTTTGGAGTC			
gr11FW	GGCTATTCAAAACAAAGTTAC	replicase p1ABAYE0001	852	
gr11RV	GTTTCCTCTCTTACACTTTT			
gr12FW	TCATTGGTATTCGTTTTTCAAAACC	replicase p2ABSDF0001	165	
gr12RV	ATTTCACGCTTACCTATTTGTC			
gr13FW	CAAGATCGTGAAATTACAGA	replicase p3ABAYE0002	780	
gr13RV	CTGTTTATAATTTGGGTCGT			
gr14FW	TTAAATGGGTGCGGTAATTT	replicase p4ABAYE0001	622	
gr14RV	GCTTACCTTTCAAAACTTTG			
gr15FW	GGAAATAAAAATGATGAGTCC	replicase p3ABSDF0018	876	
gr15RV	ATAAGTTGTTTTTGTTGTATTCG			
gr16FW	CTCGAGTTCAGGCTATTTTT	replicase repApAB49	233	
gr16RV	GCCATTTCGAAGATCTAAAC			
gr17FW	AATAACACTTATAATCCTTGTA	replicase A1s_3471	380	
gr17RV	GCAAATGTGACCTCTAATATA			
gr18FW	TCGGGTTATCACAATAACAA	replicase p2ABSDF00025	676	
gr18RV	TAGAACATTGGCAATCCATA			
gr19FW	ACGAGATACAAACATGCTCA	replicase rep135040	815	
gr19RV	AGCTAGACATTTCAGGCATT			
PCR-based sequence group (SG) identification				
Gr1_ompAF	GATGGCGTAAATCGTGGTA	*ompA* gene internal fragment	355	94ºC-3min (1 cycle);
Gr1/Gr2_ompA R	CAACTTTAGCGATTTCTGG	from SG1		94ºC-45s, 57ºC-45s, (45)
Gr1csu_EF	CTTTAGCAAACATGACCTACC	*csuE* gene internal fragment from	702	72ºC-1min (30 cycles);
Gr1_csuER	TACACCCGGGTTAATCGT	SG1		72ºC-5min (1 cycle)

Table 7 – cont.

Gr1_OXA66F **Gr1_OXA66R**	GCGCTTCAAAATCTGATGTA GCGTATATTTTGTTTCCATTC	*bla*$_{OXA-51-like}$ internal fragment from SG1	559		
Gr2_ompAF	GACCTTTCTTATCACAACGA	*ompA* gene internal fragment from SG2	343		
Gr2_csuEF **Gr2_csuER**	GGCGAACATGACCTATTT CTTCATGGCTCGTTGGTT	*csuE* gene internal fragment from SG2	580		
Gr2_OXA69F **Gr2_OXA69R**	CATCAAGGTCAAACTCAA TAGCCTTTTTTCCCCATC	*bla*$_{OXA-51-like}$ internal fragment from SG2	162		
MLST of *Acinetobacter baumannii* complex, develloped by Bartual *et al* (2005) (primers from database)					
Citrato F1 **Citrato R12**	AATTTACAGTGGCACATTAGGTCCC GCAGAGATACCAGCAGAGATACACG	amplification and sequencing of *gltA* locus	722		
APRU F **UP1E R**	TGTAAAACGACGGCCAGTGCNGGRTCYT TYTCYTGRCA CAGGAAACAGCTATGACCAYGSNGGNGG NAARTTYRA	amplification of *gyrB* locus	909		
M13 [-21] **M13 F**	TGTAAAACGACGGCCAGT CAGGAAACAGCTATGACC	sequencing of *gyrB* locus			
GDHB 1F **GDHB 775R**	GCTACTTTTATGCAACAGAGCC GTTGAGTTGGCGTATGTTGTGC	amplification of *gdhB* locus	775	94ºC-2min (1 cycle); 94ºC-1min, 55ºC-1 min, 72ºC-2min (30 cycles); 72ºC-2min (1 cycle)	(3)
GDH SEC F **GDH SEC R**	ACCACATGCTTTGTTATG GTTGGCGTATGTTGTGC	sequencing of *gdhB* locus			
RA1 **RA2**	CCTGAATCTTCYGGTAAAAC GTTTCTGGGCTGCCAAACATTAC	amplification and sequencing of *recA* locus	425		
CPN 3F2 **CPN R2**	ACTGTACTTGCTCAAGC TTCAGCGATGATAAGAAGTGG	amplification and sequencing of *cpn60* locus	479		

Table 7 (cont.)

GPI F1	AATACCGTGGTGCTACGGG	amplification and sequencing of	508		
GPI R1	AACTTGATTTTCAGGAGC	*gpi* locus			
70F RPOD	ACGACTGACCCGGTACGCATGTAYAG MGNGARATGGGNACNGT	amplification of *rpoD* locus	492		
70R RPOD	ATAGAAATAACCAGACGTAAGTTNGCY TCNACCATYTCYTTYTT				
70FS	ACGACTGACCCGGTACGCATGTA	sequencing of *rpoD* locus			
70RS	ATAGAAATAACCAGACGTAAGTT				
MLST of *Acinetobacter baumannii* complex, developed by Bartual *et al* (2005) (primers sequences kindly provided by Dr Mikail Edelstein)					
ABA-gltA-f	ACAGTGGCACATTAGGTCCC	amplification and sequencing of	720		
ABA-gltA-r	GCAGAGATACCAGCAGAGATACA	*gltA* locus			
ABA-gyrB-f	AACCATCTCAACGAAATCTTCC	amplification and sequencing of	500		
ABA-gyrB-r	GCTGGGTCTTTTTCCTGACA	*gyrB* locus			
ABA-gdh-f1	CCACATGCTTTGTTATGGGG	amplification and sequencing of	600		
ABA-gdh-r1	GATTTAAGCGTAATACTTTACCCAT	*gdhB* locus		95ºC-15min (1 cycle); 94ºC-20s, 54ºC-30s, 72ºC-45s (30 cycles); 72ºC-3min (1 cycle)	
ABA-recA-f	GGTCCTGAATCTTCTGGTAAAAC	amplification and sequencing of	400		
ABA-recA-r	GAATTTAAGAGCATTACCACCAGT	*recA* locus			
ABA-cpn60-f	CAACTGTACTTGCTCAAGC	amplification and sequencing of	450		
ABA-cpn60-r	CGCTTCACCTTCAACATCTTC	*cpn60* locus			
ABA-gpi-f	AAAATCCATGCTGGGCAATA	amplification and sequencing of	390		
ABA-gpi-r2	CAATACAAGACCAAAGAGAATAACG	*gpi* locus			
ABA-rpoD-f	GTGAAGGTGAAATCAGCATTGC	amplification and sequencing of	720		
ABA-rpoD-r	GCAATTTGTTCATCTAACCAAGC	*rpoD* locus			
MLST of *Acinetobacter baumannii*, developed by the Institut Pasteur					
cpn60F	ACTGTACTTGCTCAAGC	amplification and sequencing of	405	94ºC-2min (1 cycle);	
cpn60R	TTCAGCGATGATAAGAAGTGG	*cpn60* locus		94ºC-30s, 50ºC-30s,	(34)

Table 7 (cont.)

fusA7 **fusA8**	ATCGGTATTTCTGCKCACATYGAT CCAACATACKYTGWACACCTTTGTT	amplification and sequencing of *fusA* locus	633	72ºC-30s (35 cycles); 72ºC-5min (1 cycle)	
gltAF **gltAR**	AATTTACAGTGGCACATTAGGTCCC GCAGAGATACCAGCAGAGATACACG	amplification and sequencing of *gltA* locus	483		
pyrG7 **pyrG8**	GGTGTTGTTTCATCACTAGGWAAAGG ATAAATGGTAAAGAYTCGATRTCACCMA	amplification and sequencing of *pyrG* locus	297		
RA1 **RA2**	CCTGAATCTTCYGGTAAAAC GTTTCTGGGCTGCCAAACATTAC	amplification and sequencing of *recA* locus	372		
rplB7 **rplB8**	GTAGAGCGTATTGAATACGATCCTAACC CACCACCACCRTGYGGGTGATC	amplification and sequencing of *rplB* locus	330		
Vic4 **Vic6**	GGCGAAATGGC(AGT)GA(AG)AACCA GA(AG)TC(CT)TCGAAGTTGTAACC	amplification and sequencing of *rpoB* locus	456		
Characterization of resistance to aminoglycosides					
armA_F **armA_R**	TGCATCAAATATGGGGGTCT GGATTGAAGCCACAACCAAA	16S rRNA methylase gene	770	95ºC-3min (1 cycle); 95ºC-30s, 50ºC-1min, 72ºC-1min (30 cycles); 72ºC-10min (1 cycle)	(25) (25)
aphA6_F **aphA6_R**	ATGGAATTGCCCAATATTATTC TCAATTCAATTCATCAAGTTTTA	aminoglycoside (3') phosphotransferase APH(3')-VI (aphA6) gene	770		
Characterization of regulator genes for the efflux pump AdeABC					
adeR_F **adeR_R**	ATGTTTGATCATTCTTTTTCTTTTG TTAATTAACATTTGAAATATG	Amplification and sequencing of the regulator gene *adeR*	686	95ºC-3min (1 cycle); 95ºC-30s, 50ºC-1min, 72ºC-1min (30 cycles); 72ºC-10min (1 cycle)	(25) (35)
adeS_F **adeS_R**	ATGAAAAGTAAGTTAGGAATTAGTAAG TTAGTTATTCATAGAAATTTTTATG	Amplification and sequencing of the sensor kinase gene *adeS*	1060		
Quantitative Real-Time-PCR					
q_adeB _F	AACGGACGACCATCTTTGAGTATT	quantitation of mRNA transcripts	84	*Taq* activation step:	(35)

Table 7 – cont.

q_adeB_R	CAGTTGTTCCATTTCACGCATT	for *adeB* gene	150	95ºC-12 min 40 cycles:	
q_16S_F	CAGCTCGTGTCGTGAGATGT	quantitation of mRNA transcripts		95ºC-15s	
q_16S_R	CGTAAGGGCCATGATGACTT	for 16S rRNA gene		60ºC-1min	(35)

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6.2 Sequence submitted to GenBank

6.2.1 *Acinetobacter haemolyticus* strain AC63 plasmid pAC63, partial sequence.

GenBank: JN982951.1

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LOCUS       JN982951               5184 bp    DNA     linear   BCT 25-APR-2012

DEFINITION  Acinetobacter haemolyticus strain AC63 plasmid pAC63, partial
            sequence.
ACCESSION   JN982951
VERSION     JN982951.1  GI:384929478
KEYWORDS    .
SOURCE      Acinetobacter haemolyticus
  ORGANISM  Acinetobacter haemolyticus
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Moraxellaceae; Acinetobacter.
REFERENCE   1  (bases 1 to 5184)
  AUTHORS   Grosso,F., Quinteira,S., Novais,A. and Peixe,L.
  TITLE     The role of common blaOXA-24/40-carrying platforms and plasmids in
            the spread of OXA-24/40 among Acinetobacter spp. clinical isolates
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 5184)
  AUTHORS   Grosso,F., Quinteira,S., Novais,A. and Peixe,L.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-NOV-2011) Laboratory of Microbiology, REQUIMTE -
            Faculdade de Farmacia da Universidade do Porto, Rua Anibal Cunha,
            164, Porto 4050-047 Porto, Portugal
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6.2.2 *Acinetobacter baumannii* strain AC92 plasmid pAC92, partial sequence.

GenBank: JN982952.1

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DEFINITION  Acinetobacter baumannii strain AC92 plasmid pAC92, partial
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ACCESSION   JN982952
VERSION     JN982952.1  GI:384929486
KEYWORDS    .
SOURCE      Acinetobacter baumannii
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            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Moraxellaceae; Acinetobacter; Acinetobacter
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            complex.
REFERENCE   1  (bases 1 to 3567)
  AUTHORS   Grosso,F., Quinteira,S., Novais,A. and Peixe,L.
  TITLE     The role of common blaOXA-24/40-carrying platforms and plasmids in
            the spread of OXA-24/40 among Acinetobacter spp. clinical isolates
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 3567)
  AUTHORS   Grosso,F., Quinteira,S., Novais,A. and Peixe,L.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-NOV-2011) Laboratory of Microbiology, REQUIMTE -
            Faculdade de Farmacia da Universidade do Porto, Rua Anibal Cunha,
            164, Porto 4050-047 Porto, Portugal
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