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**CARBAPENEMASE-PRODUCING
ENTEROBACTERIACEAE: FROM COLONIZATION TO
INFECTION**

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Trabalho realizado sob a orientação da Professora Doutora Luísa Peixe (Faculdade de Farmácia, Universidade do Porto) e coorientação da Doutora Ângela Novais (Faculdade de Farmácia, Universidade do Porto).

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Ana Margarida Carvalho Guerra

*Ao meu avô,
Por nos ter ensinado a lutar.*

“Remember to look up at the stars and not down at your feet
Try to make sense of what you see and
Wonder about what makes the universe exist
Be curious”

Stephen Hawking

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“If I have seen further it is by standing on the shoulders of giants.”

Isaac Newton

ABSTRACT

The emergence and spread of resistance to carbapenems, namely due to the production of carbapenemases, leaves few therapeutic options and poses a major threat to public health. In 2015, an outbreak of KPC-producing *K. pneumoniae* (ST147-K64) was reported in a Portuguese hospital. Despite the measures implemented, the incidence of carbapenemase-producing *Enterobacteriaceae* (CPE) in this hospital has been increasing in recent years.

The aim of the present study was to understand the dynamics of CPE infection and colonization among hospitalized patients in this hospital where infection control measures and active screening have been implemented since CPE emergence, in order to improve preventive infection control decisions.

One hundred and twenty one confirmed CPE isolates were analysed. They included (i) 87 isolates from rectal swabs (February 2018) and (ii) 34 clinical isolates (November 2017 - February 2018). Relationship among isolates was established by phenotypic (Fourier Transform Infrared Spectroscopy) and genotypic (*wzi* sequencing) capsular (K) typing methods and the type of carbapenemase were identified by PCR and sequencing. For *E. coli* isolates the phylogenetic group and ST131 were identified by PCR.

K. pneumoniae (*Kp*) was the most common species isolated from both colonization and infection samples, that in most cases produced KPC(-3) (n= 92%); *E. coli* (including ST131), *E. cloacae* complex, *K. oxytoca*, and *C. freundii* complex were also found mainly in colonized patients, as well as other carbapenemases types (e.g. OXA-48, VIM). Internal medicine and women surgery were the wards with a higher number of isolates, with differences in CPE population. Most patients (79%) became colonized after their admission at this hospital, before or after infection. Patients under decolonization by faecal microbiota transplantation became recolonized. Diverse K-types were observed throughout time, though most (51%) KPC-*Kp* were linked to K-type K64 (ST147), and isolates with K-type KL27 (19%) were also frequent.

These data highlight a more diverse CPE population dispersed throughout multiple patients and units, and suggest a nosocomial acquisition in most cases. Furthermore, it underlines that established infection control measures should be reanalysed and monitored in order to reduce CPE spread among patients and prevent new clones from disseminating.

KEYWORDS: Carbapenems; Carbapenemase-producing-*Enterobacteriaceae*; infection control measures.

RESUMO

A emergência e disseminação de resistência aos carbapenemos, principalmente através da produção de carbapenemases, leva a uma escassez de opções terapêuticas e constitui uma ameaça à segurança pública. Em 2015, um surto de *K. pneumoniae* (ST147-K64) foi reportado num hospital português. Apesar das medidas de controlo de infeção implementadas, a incidência de *Enterobacteriaceae* produtoras de carbapenemases (EPC) neste hospital tem vindo a aumentar nos últimos anos.

O objetivo do presente estudo foi compreender as dinâmicas de colonização e infeção por EPC em doentes internados neste hospital, onde as medidas de controlo de infeção e rastreio ativo foram implementadas desde a sua emergência, de modo a melhorar as decisões de controlo de infeção.

Foram analisados cento e vinte e um isolados de *Enterobacteriaceae* produtores de carbapenemase. Estes incluíam (i) 87 isolados obtidos a partir de exsudados rectais (fevereiro 2018) e (ii) 34 isolados de amostras clínicas (novembro 2017 - fevereiro 2018). A relação entre isolados foi estabelecida por métodos de tipagem capsular (K) fenotípicos (espectroscopia de Infravermelhos com Transformada de Fourier) e genotípicos (sequenciação de *wzi*); o tipo de carbapenemases foi identificado por PCR e sequenciação. Nos isolados de *E. coli*, identificou-se o grupo filogenético e ST131 por PCR.

K. pneumoniae (Kp) foi a espécie mais frequentemente isolada das amostras de colonização e de infeção, sendo na maioria dos casos produtora de KPC(-3) (n= 92%); *E. coli*, (incluindo ST131), *E. cloacae* complex, *K. oxytoca* e *C. freundii* complex foram também encontrados, principalmente em doentes colonizados, assim como outros tipos de carbapenemase (p. ex., OXA-48, VIM). Medicina interna e cirurgia das mulheres foram as áreas com maior número de isolados, apresentando diferenças na população de EPC. A maior parte dos doentes (79%) ficou colonizado após a admissão neste hospital, antes ou depois da ocorrência de infeção. Nos doentes em descolonização por transplante fecal observou-se uma recolonização. Vários K-tipos foram observados ao longo do tempo, apesar da maior parte (51%) de KPC-Kp estar associada ao K-tipo K64 (ST147) e isolados com K-tipo KL27 (19%) serem também frequentes.

Estes dados evidenciam uma população de EPC mais diversa por todos os doentes e unidades, e sugerem uma aquisição nosocomial. Realçam que as medidas de controlo de infeção estabelecidas devem ser reanalisadas e monitorizadas, de forma a reduzir a disseminação de EPC entre os doentes e evitar a disseminação de novos clones.

PALAVRAS-CHAVE: Carbapenemos; *Enterobacteriaceae* produtoras de carbapenemase; medidas de controlo de infeção.

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LIST OF ABBREVIATIONS

AbR – Antibiotic Resistant

CDC – Centers for Disease Control and Prevention

CPE – Carbapenemase Producing *Enterobacteriaceae*

CRE – Carbapenemase Resistant *Enterobacteriaceae*

ESBL – Extended Spectrum β -lactamase

ESCMID - European Society of Clinical Microbiology and Infectious Diseases

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FTIR – Fourier Transform Infrared

IMP – Imipenemase β -lactamase

KPC – *Klebsiella pneumoniae* carbapenemase

K-Type – Capsule Type

MDR – Multidrug resistant

MGE – Mobile genetic element

NDM – New Delhi metallo- β -lactamase

OMP – Outer membrane protein

OXA – Oxacillinase

PBP - Penicillin Binding Protein

PCR – Polymerase Chain Reaction

ST – Sequence Type

VIM – Verona integron-borne metallo- β -lactamase

WHO – World Health Organization

CHAPTER 1 - INTRODUCTION

1.1. ANTIBIOTIC RESISTANCE CRISIS

Infectious diseases were the leading cause of death in the world at the beginning of the twentieth century, until the discovery of penicillin by Sir Alexander Fleming in 1928 (2-5). Since then, antibiotics have saved millions of lives and enabled important medical procedures. The “golden era” of antibiotic discovery and clinical development took place in the period between 1940 and 1960, which led to confidence that infectious diseases can be controlled and prevented (2, 5). However, some pathogens soon became resistant to many of those drugs (figure 1) and now antibiotic resistance has been seen to nearly all antibiotics developed (3, 5).

Antibiotic resistance is now a worldwide problem. A study from the Centers of Disease Control and Prevention (CDC, Atlanta) estimated that at least 23000 people died each year in the USA as a direct result of infections caused by antibiotic resistant (AbR) bacteria (6). On this trajectory, as many as 10 million people a year could die from AbR bacteria worldwide by 2050, at a cost of \$100 trillion (7). Infectious diseases are the second cause of death in the world, and the third in developed nations (4, 5). In addition to increasing morbidity and mortality, these infections are also a major economic burden to the healthcare systems, as they require significantly longer hospital stays and additional doctor visits (6). As such, in 2013, the CDC described this antimicrobial resistance as one of our most serious health threats and, in 2014, the World Health Organization (WHO) alerted for the real possibility of a post-antibiotic era, in which a common infection could kill (3, 4).

The problem of antimicrobial resistance led to the need to create harmonized definitions with which to classify bacteria that are resistant to numerous antimicrobial agents: multidrug resistant (MDR), extreme drug resistant (XDR) or pandrug resistant (PDR) (figure 2). The MDR category applies with non-susceptibility to at least one agent in three or more antimicrobial categories. XDR implies non-susceptibility to at least one agent in all but two or fewer antimicrobial categories. A PDR strain exhibits non-susceptibility to all agents in all antimicrobial categories (8).

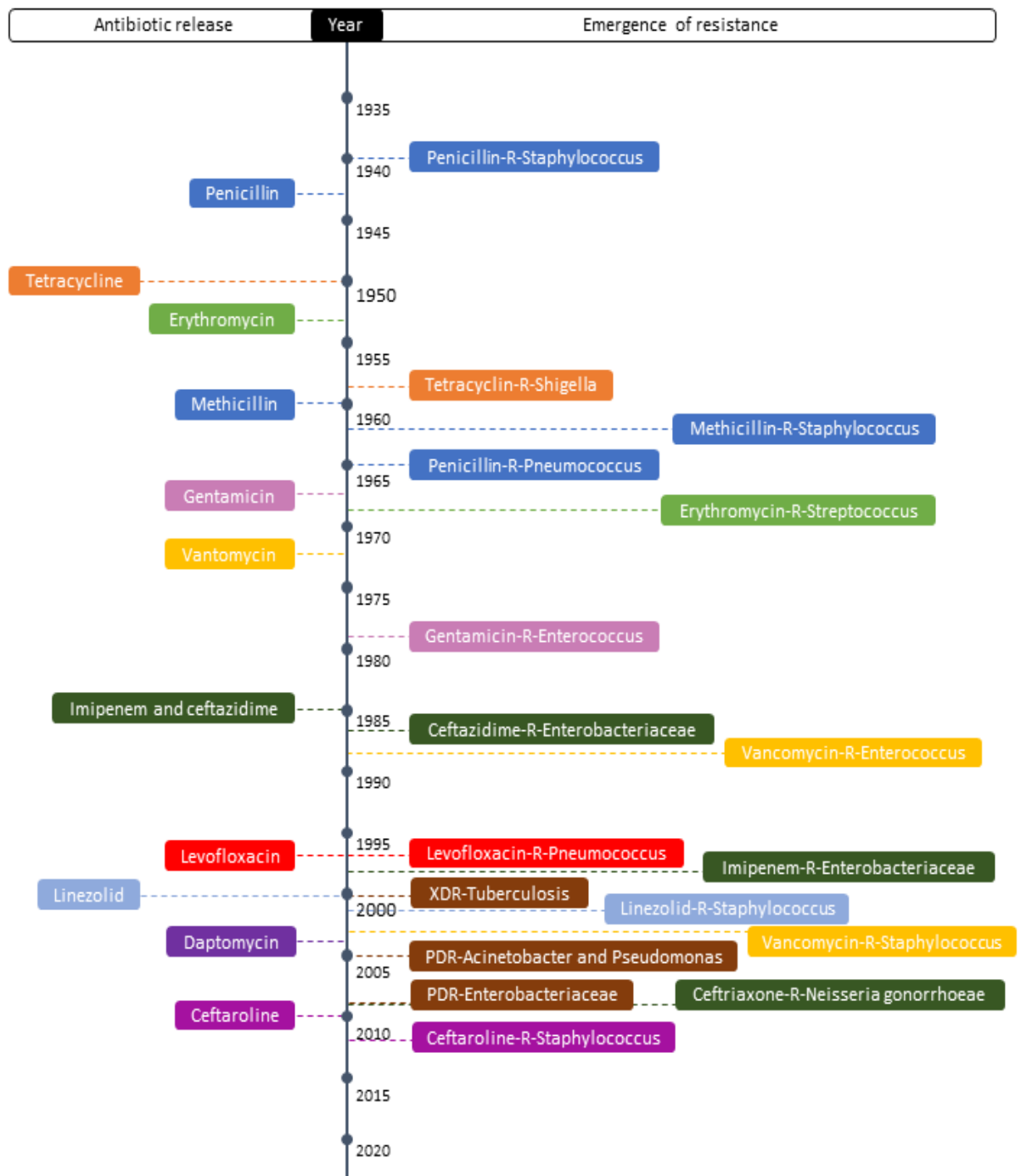


Figure 1- Timeline of antibiotic resistance showing that the introduction of each antibiotic was quickly followed by an adaptation of bacteria. Adapted from (1).

Infections caused by MDR bacteria commonly occur in hospitalized patients, due to the extensive use of antibiotics and the need of invasive procedures (4). However, some MDR bacteria have become quite prevalent in community-acquired infections as well (9). For example, methicillin-resistant *Staphylococcus aureus* (MRSA) was initially limited to hospital-acquired infections until 1980, when it spread throughout the community (10).

Antibiotic resistance among Gram-negative bacteria is particularly worrisome because they are becoming resistant to nearly all drug options available (4). The greatest concern is caused by *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter cloacae* complex and *Escherichia coli* pathogenic and MDR strains. These bacteria can cause several infections, ranging from urinary tract to bloodstream infections or severe pneumonias. Currently, *K. pneumoniae* is one of the most frequently isolated organisms in intensive care unit infections (6).

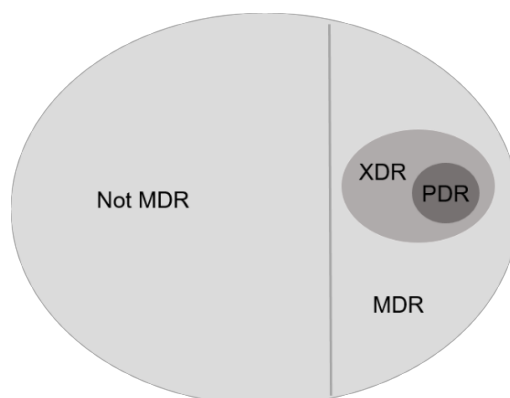


Figure 2 - Relationship between MDR, XDR and PDR to each other. Adapted from (8).

In face of the rapid emergence of antimicrobial resistant human pathogens there is a global need for new antibiotics. Nevertheless, after the successful discovery of antibiotics in the past, the pipeline is now running dry, as there has been a paucity of new compounds being developed or introduced (3, 7).

1.1.1. CAUSES OF ANTIBIOTIC RESISTANCE CRISIS

The factors that contribute to the emergence and dissemination of AbR bacteria are the following: (i) overuse of antibiotics for human or veterinary infections; (ii) extensive agricultural use; (iii) inefficient infection control practices; (iv) a scarcity of new antimicrobial drugs developed in the last years; and (v) worldwide travel and trade of food and goods (6).

The overuse of antibiotics is the major factor leading to antibiotic resistance around the world and has been reported a direct relationship between antibiotic consumption and the emergence of AbR bacteria (4, 6). Antibiotics are overprescribed by doctors, being among the most commonly prescribed drugs in human medicine (4). However, up to 50% of all the antibiotics prescribed are not needed and in several cases the duration of therapy or the choice of agent is incorrect. Besides that, in many countries antibiotics are underregulated and available to buy over the counter without a prescription, which promotes overuse (1). In addition, antimicrobial drugs remain in wide use within animal context (6),

increasing the risk of emergence or selection of antimicrobial resistance and further transmission through the food chain (4).

The prevalence of AbR human pathogens is rapidly increasing, but the discovery and development of new antimicrobial drugs have slowed dramatically (figure 3), with most of pharmaceutical companies abandoning the antibiotic field (4, 7, 10). At this moment, few antibacterial drugs with activity against Gram-negative bacteria are being developed, due to a combination of discovery, clinical, regulatory and economic obstacles (11).

The development of antimicrobial drugs is no longer considered an economically wise investment, as they are short-course therapies and therefore not as profitable as medicines for chronic conditions (7). Furthermore, changes in requirements for clinical trial design have made the approval of new antibiotics more challenging and with higher costs (7, 11).

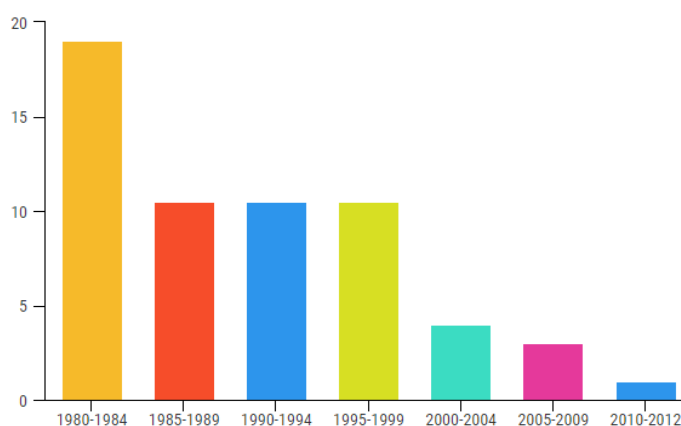


Figure 3 - Number of Antibacterial Drug Application (NDA) Approval *per* Years Intervals. Adapted from (5).

1.2. ANTIBIOTIC RESISTANCE THREATS

In 2013, the CDC has prioritized drug-resistant bacteria based on the level of concern into three categories: *urgent*, *serious* and *concerning*. Pathogens categorized as urgent threats to public health are *Clostridium difficile*, Drug-resistant *Neisseria gonorrhoeae* and Carbapenem-resistant *Enterobacteriaceae* (CRE) (2, 4).

Enterobacteriaceae, such as *K. pneumoniae*, are a family of Gram-negative bacteria responsible for a variety of community and healthcare-associated infections (12). Carbapenems are β -lactam antibiotics typically considered agents of last resort, used for the treatment of severe infections caused by MDR-*Enterobacteriaceae*. However, over the past ten years CRE have been increasingly reported worldwide. Infections caused by CRE are associated with poor clinical outcomes and high mortality, leading to failure in first line

therapy and to very limited therapeutic options (13). CDC estimated that more than 9000 healthcare-associated infections are caused by CRE each year in the United States, resulting in 600 deaths per year in this country (6).

CRE were initially defined as *Enterobacteriaceae* that are nonsusceptible to a carbapenem (14). However, resistance to carbapenems in *Enterobacteriaceae* may result either from expression of carbapenemases or by combined effects of AmpC-type or extended-spectrum β -lactamases (ESBLs) and decreased outer membrane permeability (13). Because of that, in 2015, the CDC revised this definition of CRE to any *Enterobacteriaceae* which are resistant to any carbapenem (i.e., minimum inhibitory concentrations of > 2 mg/L for doripenem, > 8 mg/L meropenem or imipenem OR > 1 mg/L for ertapenem) or are documented to produce a carbapenemase. In addition, for those *Enterobacteriaceae* which may have intrinsic reduced susceptibility to imipenem, such as *Proteus mirabilis*, resistance to a non-imipenem carbapenem is required (14, 15).

Carbapenem-resistant *Enterobacteriaceae* strains that do not produce carbapenemases (non-CP CRE) are considered to be much less important from a public health perspective than carbapenemase producers (CP-CRE), though occasional outbreaks have been reported (16). The spread of carbapenemase producers is one of the most worrying issues among antibiotic resistance in Gram-negatives, from either a clinical and epidemiological perspective (17).

1.3. MECHANISMS OF RESISTANCE TO CARBAPENEMS

The β -lactam group of antibiotics includes penicillins, cephalosporins, carbapenems, and monobactams (18). They act to inhibit bacterial cell wall synthesis by the following steps: binding of the drug to the penicillin-binding proteins (PBP) located in the bacterial cytoplasmic membrane; inhibition of the transpeptidation reaction that crosslinks the linear peptidoglycan chain constituents of the cell wall; and activation of autolytic enzymes that cause lesions in the bacterial cell wall (19).

Primary mechanisms of carbapenem resistance in *Enterobacteriaceae* include: (1) enzymatic inactivation of the antibiotic by chromosome or plasmid-encoded enzymes with hydrolytic activity against β -lactam molecules; (2) decreased outer membrane permeability through production of modified porins, loss of porin expression, or a shift in the types of porins found in the outer membrane; and (3) efflux of the antibiotic to the outside of the bacterium through production of an efflux pump (figure 4) (13).

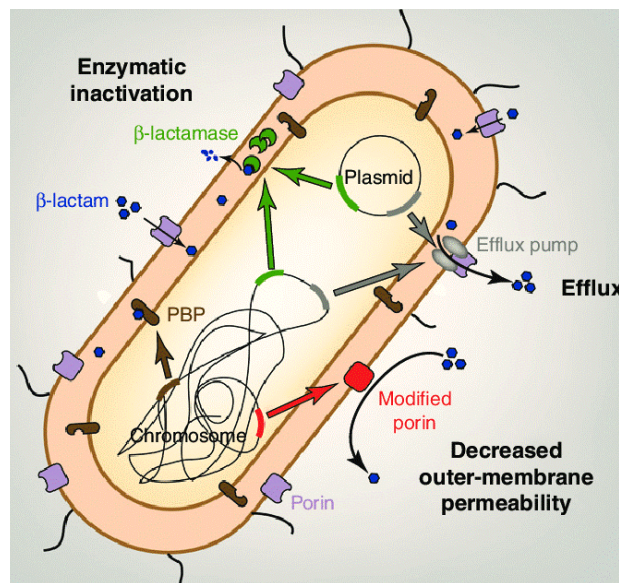


Figure 4 - Primary mechanisms of β -lactam resistance in *Enterobacteriaceae*. Adapted from (13)

1.3.1. PRODUCTION OF β -LACTAMASES

The most common mechanism of carbapenem resistance in *Enterobacteriaceae* is the production of β -lactamases (18). These enzymes hydrolyse the β -lactam ring in the periplasmic space, therefore preventing PBP inhibition (figure 5) (20).

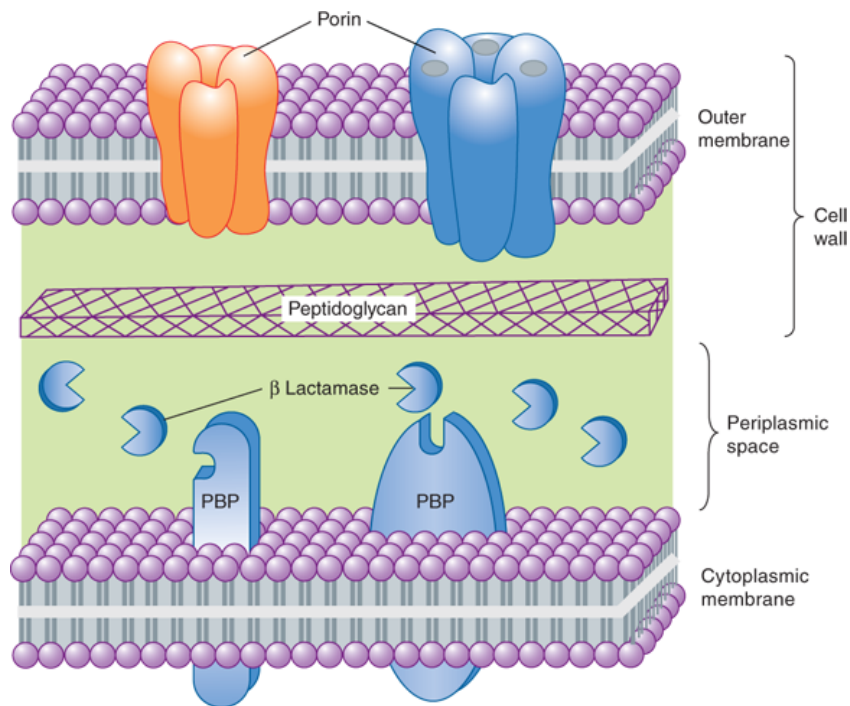


Figure 5 - Mechanism of action of beta-lactams. Adapted from (19).

Genes encoding for β -lactamases, *bla* genes, can be found in the chromosome or in mobile genetic elements, which contributes to their wide dissemination (21).

The destruction of antibiotics by the action of β -lactamases has been a major cause of antibiotic resistance since the discovery of a plasmid-encoded penicillinase, present in penicillin-resistant *S. aureus* in 1940. From then on, the development of new β -lactams compounds has been followed by the appearance of new enzymes (figure 6).

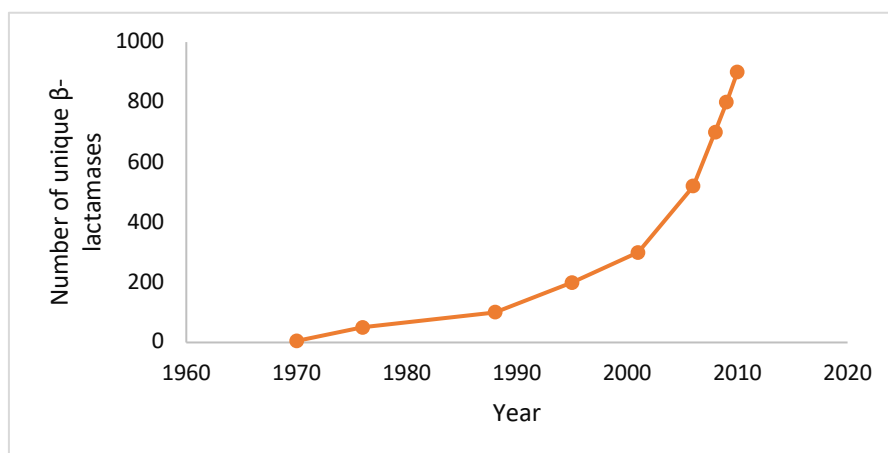


Figure 6 - Number of β -lactamase enzymes identified since the introduction of the first β -lactam antibiotics. Adapted from (22).

The penicillinases were followed by ESBLs that inactivate oxyimino-cephalosporins (23). In some instances, porin loss in ESBL-producing strains increases resistance to fourth-generation cephalosporins and carbapenems (24). As the prevalence of ESBL and plasmid-borne cephalosporinases increased, so did the consumption of carbapenems, which promoted the emergence and dissemination of carbapenemase-producing *Enterobacteriaceae* (CPE) through the diffusion of plasmid-borne carbapenemases (20). They comprise a variety of hydrolysing enzymes with the ability to inactivate a broad range of β -lactams, including penicillins, cephalosporins, monobactams, and carbapenems (23).

To date, thousands of β -lactamases have been identified (<http://www.bldb.eu>) and there are two main classification schemes (table 1). The most frequently used is the Ambler classification scheme, which separates β -lactamases into four groups (A, B, C and D) based on amino acid sequence identity. According to this classification, β -lactamases of classes A, C and D have a serine residue in the active site, whereas class B enzymes require zinc for their activity. The other classification is the one proposed by Bush-Jacoby, which relies on substrate specificity and their inactivation by classical β -lactamase inhibitors to divide β -lactamases into four groups, each with many subgroups (21, 25).

- **Group 1 / Ambler Class C:** Cephalosporinases that are encoded on the chromosomes of many *Enterobacteriaceae* and that are not inhibited by the classical β -lactamase inhibitors (clavulanic acid, tazobactam). When produced in large amounts they can provide resistance to carbapenems (25).
- **Group 2 / Ambler Classes A and D:** This is the largest group of β -lactamases and is constituted by several subgroups. It comprises β -lactamases with a hydrolytic activity over penicillins, extended-spectrum cephalosporins (ESBLs), monobactams, cloxacillin and carbapenems.
 - The subgroup 2f contains the KPC enzymes from the molecular class A (25). Class A enzymes hydrolyse penicillins, cephalosporins, carbapenems and aztreonam. Their hydrolytic activity *in vitro* is inhibited by clavulanic acid and tazobactam (13). Other class A enzymes with carbapenemase activity are some GES type β -lactamases variants (GES-2, -4, -5, -6). Their hydrolysis spectrum includes penicillins, extended-spectrum cephalosporins and carbapenems. They remain susceptible to classic β -lactam inhibitors.
 - In the subgroups 2d, 2de and 2df are the OXAs enzymes, corresponding to the molecular class D. They do not generally

hydrolyse extended-spectrum β -lactams or aztreonam but are active against oxacillin. They also confer high resistance to temocillin, which can be used for phenotypic identification of OXA-48 producers, since the other classes of carbapenemases are susceptible to this drug (26). These enzymes are not inhibited by either clavulanic acid or EDTA, but rather by NaCl (13).

- **Group 3 / Ambler Class B:** Includes the MBLs, a unique group of β -lactamases that are dependent on zinc ions for activity rather than the active-site serine found in the other classes. They hydrolyse carbapenems but have a poor affinity for monobactams. They are inhibited by chelating agents, such as EDTA, but not by classical β -lactamase inhibitors (25). Despite the lack of activity against monobactams, the presence of additional β -lactamases (e.g. ESBL) tends to compromise the efficacy of these agents (24).
- **Group 4:** Included enzymes that were incompletely characterized but the most recent review of this classification scheme has omitted this group. (25)

Table 1- Classification schemes for β -lactamases. Adapted from (25)

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

Among the classes of β -lactamases defined by the Ambler classification system, the ones that confer carbapenem resistance in *Enterobacteriaceae* belong to three of them: Class A, Class B and Class D (table 2) (27).

Table 2- Main carbapenemases in *Enterobacteriaceae*. Adapted from (13).

Ambler class	Enzyme	Plasmid/ Chromosome	Hydrolysis spectrum						Inhibitor
			Penicillins	1st generation cephalosporins	2nd generation cephalosporins	3rd generation cephalosporins	Aztreonam	Carbapenems	
A	SME-1 to -3	Chromosome	++	++	--	+	+	+	Clavulanate, tazobactam, sulfabactam, NXL-104
	NMC-A	Chromosome	++	++	--	+	--	++	
	IMI-2	Plasmid	++	++	--	+	--	++	
	GES-4,-5,-6	Plasmid	++	++	+	+	--	+	Clavulanate, tazobactam, boronic acid, sulfabactam
	KPC-2 to -12	Plasmid	++	++	--	++	+	++	
B	IMP-1 to -33	Plasmid	++	++	++	++	--	++	EDTA
	VIM-1 to -33	Plasmid	++	++	++	++	--	++	
	NDM-1 to -6	Plasmid	++	++	++	++	--	+	
	KHM-1	Plasmid	++	++	++	++	--	++	
D	OXA-48	Plasmid	++	++	+/-	+/-	--	+	NaCl
	OXA-181	Plasmid	++	++	+/-	+/-	--	+	

1.3.2. PORIN PERMEABILITY CHANGES

Carbapenem resistance was first described in *Enterobacter* spp. isolates overexpressing a chromosomal *ampC* gene encoding an intrinsic cephalosporinase and exhibiting modifications in their outer membrane protein (OmpC or OmpF) porins. This mechanism of carbapenem resistance has also been observed in other species that do not express an intrinsic cephalosporinase, such as *E. coli* and *K. pneumoniae*. In those cases, resistance is due to decreased cell membrane permeability owing to modifications in OmpK35 and OmpK36 for *K. pneumoniae* and in OmpF and OmpC for *E. coli*, in most cases combined with expression of plasmid-encoded AmpC or ESBL (13).

1.3.3. EFFLUX

Overexpression of AcrA, a component of AcrAB-TolC efflux system, was described as responsible for imipenem resistance in *Enterobacter aerogenes* (13, 24).

1.4. EPIDEMIOLOGY OF CARBAPENEMASES

The first carbapenemase identified in *Enterobacteriaceae* was the chromosomally encoded NmcA from an *E. cloacae* clinical isolate in 1993 (13, 28). Nevertheless, carbapenem resistance in *Enterobacteriaceae* had been a negligible phenomenon until a carbapenem resistant *K. pneumoniae* strain was identified in 1996, in the United States, harbouring a plasmid-encoded resistance gene (*Klebsiella pneumoniae carbapenemase*, KPC). Since that time, numerous CPE have been reported and widely disseminated (28).

1.4.1. CLASS A CARBAPENEMASES

Class A carbapenemases include the enzymes KPC, GES and IMI-types, as well as nonmetallocarbapenemase type A (NMC-A) and SME enzymes, which may be found in *E. cloacae* and *Serratia marcescens*, respectively. While some carbapenemases are chromosome-encoded (e.g. IMI-1, NMC-A, SME enzymes, SHV-38 and SFC-1), others are plasmid-encoded (e.g. KPC, GES and IMI-2). Among these, KPC enzymes are the most widely spread, whereas the others are more sporadically reported or are confined to certain bacterial species or geographic regions (29).

KPCs have been the most clinically significant class A carbapenemases since their first identification (KPC-2) in 1996 from a *K. pneumoniae* strain in a North Carolina hospital (USA) (12, 29). Since then, several KPC variants (KPC-2 to KPC-24) have been identified, being KPC-2 and KPC-3 the most widespread variants (30). Within a few years, KPC producers had spread across the United States and all over the world (28).

KPC production have been reported mostly from nosocomial *K. pneumoniae* isolates and to a much lesser extent from *E.coli*, *Enterobacter* spp. and from other enterobacterial species (28). Although community-acquired KPC-producing strains have been reported in the USA and Israel a few years ago their incidence remains low (13).

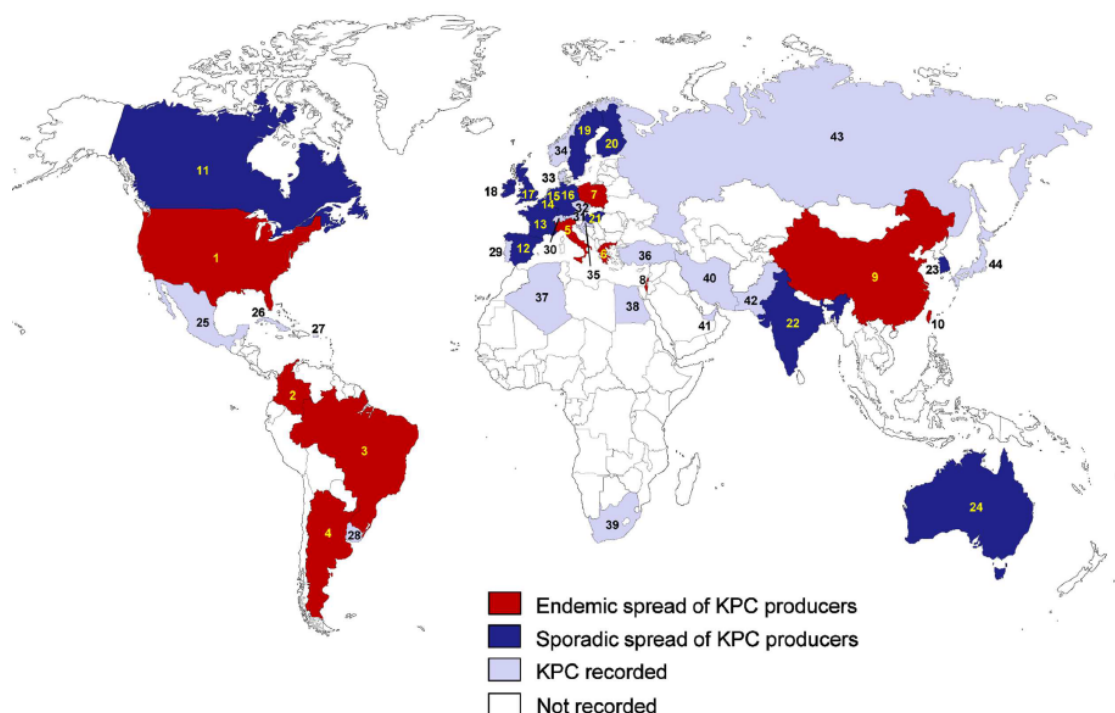


Figure 7 - Epidemiology of KPC (31). (1) USA; (2) Colombia; (3) Brazil; (4) Argentina; (5) Italy; (6) Greece; (7) Poland; (8) Israel; (9) China; (10) Taiwan; (11) Canada; (12) Spain; (13) France; (14) Belgium; (15) Netherlands; (16) Germany; (17) UK; (18) Ireland.

The epidemiology of KPC-producing *K. pneumoniae* varies geographically (figure 7) but they are currently endemic in Israel, Greece, Latin America, Italy, China, Poland, Taiwan and the USA (12, 31). Outbreaks of KPC-producers have been reported in many European countries (KPC-2-producing *K. pneumoniae* in Greece (32, 33) and KPC-3-producing *K. pneumoniae* in Italy (34), for example), especially due to patients travelling to endemic areas (31). The rapid and worrisome spread across multiple continents and species has been associated with the dissemination of a specific *K. pneumoniae* clone (ST258) expressing the genes *bla*_{KPC-2} or *bla*_{KPC-3} (28, 31). It remains the predominant ST in the USA and other countries, whereas in certain EU countries other (and diverse) *K. pneumoniae* lineages have been linked to KPC emergence and/or spread (28, 35). On the contrary, ST11 is the predominant clone observed in Asia, and has also been reported in Latin America (31). In Portugal, there was only one report of KPC-2 that was identified in an environmental *E. coli* isolate in 2010, whereas KPC-3 was first detected in 2009 in a paediatric unit of a central hospital, from 2 strains of *K. pneumoniae* belonging to ST11 (30, 36). However, some hospital outbreaks revealed the emergence of the carbapenemase KPC-3 in Portugal (35), involving isolates from diverse clones (ST11, ST14, ST15 and ST147) (30).

The *bla*_{KPC} genes are most frequently associated with a common genetic element (transposon Tn4401) (28). Tn4401 is a Tn3-like transposon (13) which has six isoforms

differing by deletions upstream of the *bla*_{KPC} gene, leading to different expression levels of the *bla*_{KPC} gene (figure 8) (30, 31).

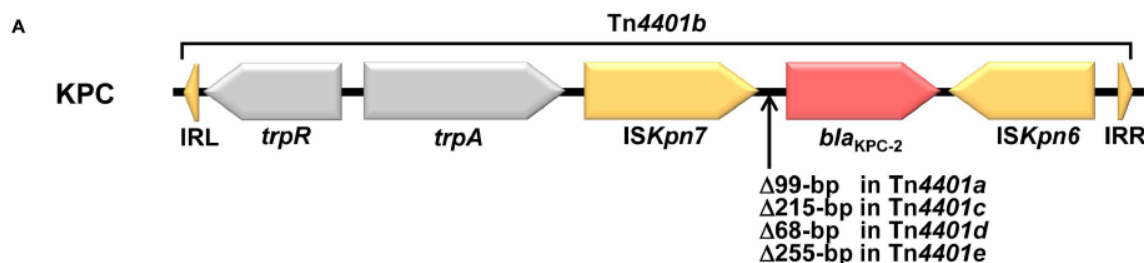


Figure 8 - The *bla*_{KPC-2} containing Tn4401 transposon. Adapted from (31)

The *bla*_{KPC} has been reported on numerous plasmid types, but the most predominant belongs to the IncF group, and particularly FII_K replicon types in *K. pneumoniae*. This plasmid type often contains several additional genes responsible for resistance to other antibiotics (31).

1.4.2. CLASS B β-LACTAMASES

Class B carbapenemases were first detected in the 1960s. However, in the 1990s the spread of MGEs harbouring genes encoding metallo-β-lactamases was responsible for increasing reports of clinical infections and nosocomial outbreaks (37). The most important types from a clinical and epidemiological point of view are the IMP-, VIM- and NDM-type enzymes (28, 37). They are usually associated with integrons carrying additional resistance genes that encode other carbapenemases, AmpC cephalosporinases or ESBLs or proteins conferring resistance to other families of antibiotics (24).

The first acquired MBL, IMP-1 (Imipenemase), was detected in *S. marcescens* strain, in Japan in 1991 (37). The gene *bla*_{IMP-1} has since then become a widespread carbapenem resistant determinant with at least 52 variants described worldwide, found in *Enterobacteriaceae*, and also *Pseudomonas* spp. and *Acinetobacter* spp. (12, 21). Nevertheless, IMP-type MBL-containing *Enterobacteriaceae* are endemic in Japan and Taiwan, with occasionally sporadic outbreaks in other countries (12). Analysis of the *bla*_{IMP} genetic environments most often revealed features of class 1 integrons, which also contained genes encoding resistance to other antimicrobials (aminoglycosides, class D β-lactamases and chloramphenicol) (12).

The second acquired MBL detected was VIM (Verona integron-encoded metallo-β-lactamase), isolated initially on *P. aeruginosa* in the late 1990s in Europe (12). VIM-1 was first identified in Verona, Italy, in 1997, and shortly after, the VIM-2 was reported in

Marseilles, France (13). Among the 46 variants described, VIM-2 is the most widely distributed MBL (12). The endemic spread of VIM-type enzymes has been reported in Greece (figure 9), where *K. pneumoniae* and *E. coli* harbouring *bla*_{VIM-1} are the predominant species (12, 28).

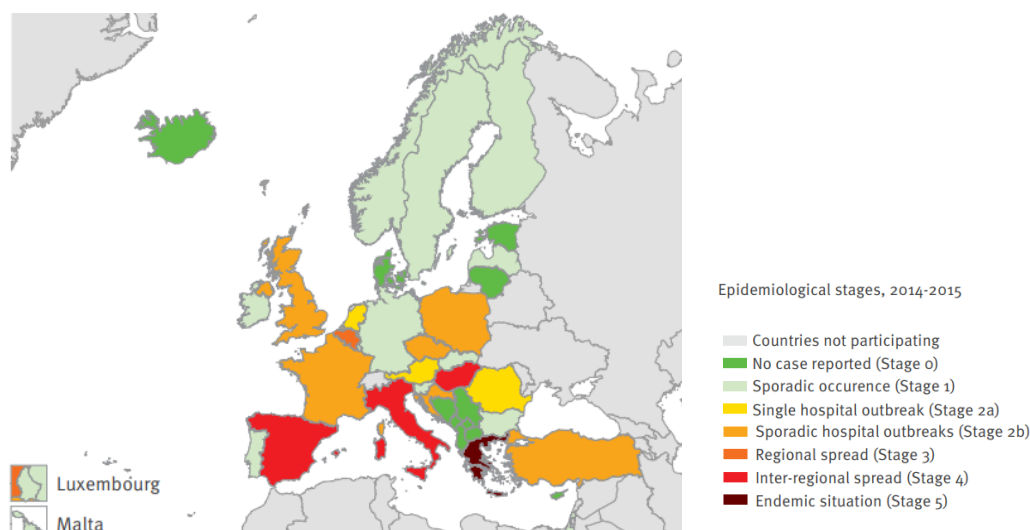


Figure 9 - Geographic distribution of VIM-producing Enterobacteriaceae in Europe. Adapted from (38).

The NDM is now one of the most clinically significant carbapenemase (31). NDM-1 was first identified in 2008, in a Swedish patient previously hospitalized in New Delhi, India (15). Since then, there has been a global dissemination of NDM MBLs (figure 10), often in patients with a history of tourism or healthcare travel in the Indian subcontinent (12, 29). NDM-type CPE have been identified on all continents, nevertheless India and Pakistan are the countries with the higher prevalence (27), where NDM producers have been found in the Indian tap and seepage water, suggesting their widespread in the environment (39).

NDM-producing bacteria are occurring both in healthcare settings and the community (12). Since its discovery, 15 NDM variants have been found and 3 more have been assigned accession numbers (40), most of them originated from Asia (31).

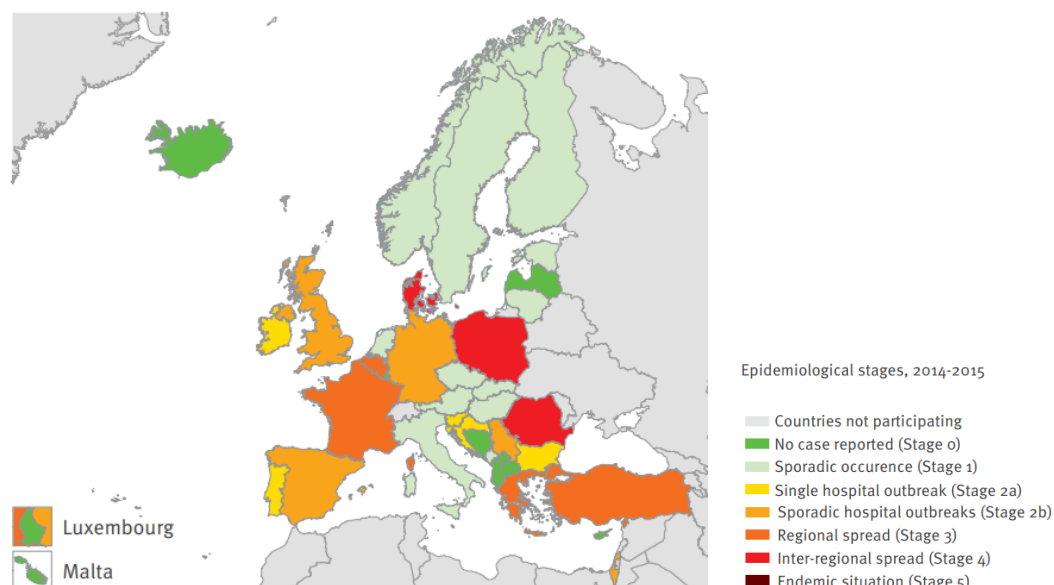


Figure 10 - Geographic distribution of NDM-producing *Enterobacteriaceae* in Europe. Adapted from (38)

The *bla_{NDM-1}* gene, unlike other carbapenemases genes, is associated with nonclonally related isolates and species (28). The presence of NDM-1 is usually accompanied by a MDR-phenotype, because plasmids carrying the *bla_{NDM-1}* gene are diverse and can harbour a high number of resistance genes encoding additional carbapenemases (21, 28). The *bla_{NDM}* gene is often observed in the transposon Tn125, flanked by two IS*Aba*125 elements (31, 41).

Of great concern, NDM-1 has been identified in *E. coli* ST131, recognized as a source of community-associated infections (15, 28). Thus, this may increase the risk of drug-resistant strains being released into the environment and further spread among humans (28).

1.4.3. CLASS D β -LACTAMASES

Only a few class D β -lactamases or oxacillinases (OXA) possess carbapenemase activity. In *Enterobacteriaceae*, OXA-48 is the most frequent class D carbapenemase, commonly reported from *K. pneumoniae* and *E. coli* isolates (31).

OXA-48 was first identified in 2003 in a *K. pneumoniae* strain, in Turkey, where it has become endemic. Since then, outbreaks have been reported from several other countries (figure 11), including Morocco, Libya, Egypt, Tunisia and India (12, 31).

OXA-48 producers are mostly identified in *K. pneumoniae* and *E. coli*, but also in various *Enterobacteriaceae* (28). A single IncL group plasmid (pOXA-48a) is the main source of the *bla_{OXA-48}* gene disseminated in a variety of enterobacterial species (28, 31).

A high related enzyme, OXA-181 (differing from OXA-48 in one aminoacid mutation) was first identified in India (28) and, since then, has been reported in many different countries (31).

The OXA-48-type producers are probably the most difficult carbapenemase producers to identify, because the isolates producing OXA-48 and OXA-181 usually present reduced susceptibility to carbapenems (13, 28). Therefore, the prevalence of these enzymes may be underestimated (12, 29).

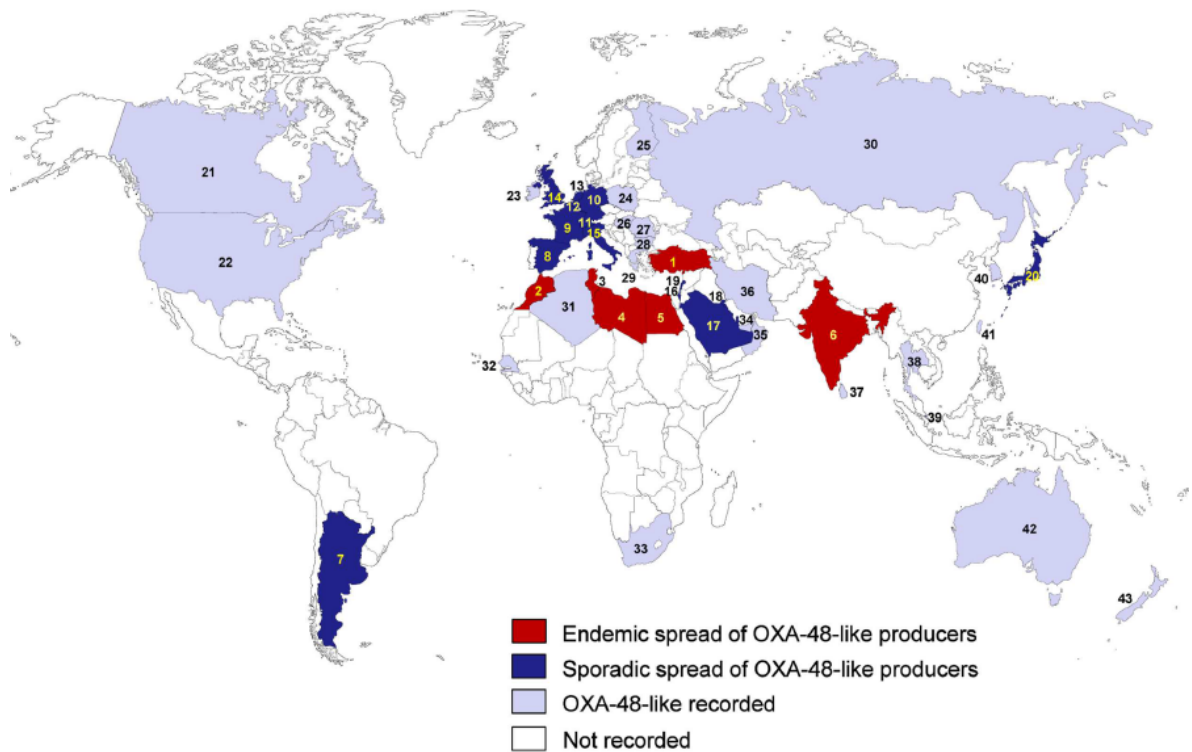


Figure 11 - Epidemiology of OXA-48-like producing *K.pneumoniae*

The prevalence of CPE varies geographically, but currently the most common species of *Enterobacteriaceae* harbouring transmissible carbapenemase genes is *K. pneumoniae* (29). CPE is seen predominantly in healthcare associated infections, with the exception of the Indian subcontinent, where community-associated infections linked with NDM-producing bacteria are common (42). Furthermore, some studies worldwide have demonstrated the presence of carbapenemase-encoding genes in *Enterobacteriaceae* from livestock (43), companion animals (44, 45) and wildlife (46).

1.5. CPE IN HEALTHCARE SETTINGS

1.5.1. RISK FACTORS FOR CPE INFECTIONS

In healthcare settings, intensive care units (ICUs) are the main reservoirs of Gram-negative multidrug resistant bacteria and *K. pneumoniae* is one of the most common pathogens found there (47).

The acquisition of CPE by patients can occur through two different modes: horizontal transmission (from another patient, through healthcare staff, from the proximal environment or through shared equipment) or *de novo* emergence of resistance, in which carbapenem susceptible isolates within a patient are genetically altered (48). Acquisition of CPE via exogenous exposure during hospitalization is the most common pathway. The introduction of CPE into the hospital environment occurs through patients who are colonized or infected, leading to healthcare worker or environmental contamination and subsequent spread to other patients (49). Recent European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Guidelines have identified the contaminated hands of healthcare workers as the interpatient main route of CPE transmission (50). Subsequent dissemination of CPE can result from contamination of the healthcare environment, like medical equipment or hospital surfaces. It is generally accepted that *K. pneumoniae* exhibits a greater propensity for environmental contamination and persistence than other *Enterobacteriaceae* (49). Multiple studies have investigated risk factors for infection by CPE, such as KPC-producing *Enterobacteriaceae*. These factors include previous ICU stay, prolonged hospitalization, critical illness, use of invasive medical devices (catheter, surgical drain), mechanical ventilation, comorbid conditions, recent antibiotic exposure and previous colonization by a CPE strain (12, 50). Important group risks for CPE colonization and persistent carriage includes long-term care facility residence, mediated by cross transmission between patients, and a poor functional status (50). Antibiotic exposure is a risk factor for CPE colonization or infection, as it may drive a selective pressure that enriches pre-existing CPE above the threshold of detection. The antibiotic exposure may increase susceptibility to CPE colonization by disrupting the intestinal microbial ecology, thereby increasing the risk of environmental or personnel contamination and subsequent horizontal transmission to other patients (49). Also, antibiotic exposure may cause bacterial genomic instability, ensuring that if advantageous *de novo* mutations arise, they become entrenched in the enteric microbiota (49). Some studies have established an association with CRE colonization or infection with previous therapy with certain antibiotics, like carbapenems, fluoroquinolones, vancomycin and metronidazole (12, 49, 50).

1.5.2. PREVENTIVE MEASURES

CPE are a public health threat that requires urgent actions and infection control measures. To avoid the transmission of CPE, the CDC recommends screening of patients, and early identification and isolation of colonized or infected patients, followed by implementation of infection control measures (12, 14, 50).

According to the ESCMID guidelines, the strongest intervention to prevent interpatient transmission seems to be hand hygiene (50-52). But it seems to be insufficient, and hand hygiene adherence should be monitored and communicated to front line staff (14).

Based on many studies, other additional measures include isolation of colonized or infected patients, cohorting of patients and medical personnel, educational campaigns, minimizing the use of invasive devices, shortening the duration of mechanical ventilation, promotion of antimicrobial stewardship and daily baths with 2% chlorhexidine impregnated wipes (12, 50, 52). The latter has been used as part of a multifaceted intervention to decrease CPE load on patient's skin and contamination of health worker's hands and environmental surfaces, as chlorhexidine gluconate works as an antiseptic agent with broad antimicrobial activity. However, the impact of this measure on KPC transmission is still not very clear (51).

Active screening is an important measure that allows the early identification of patients colonized at hospital admission or during hospitalization and the subsequent cohorting of CPE carriers (12, 50). Rectal swab or stool is the preferred specimen because *Enterobacteriaceae* form part of the intestinal microbiota. They are typically plated on selective medium, such as chromogenic media, like chromID® CARBA SMART Agar (bioMérieux) or mSuperCARBA™ (CHROMagar™). These media contain a mixture of antibiotics enabling the selective growth of carbapenem resistant strains, and chromogenic substrates that allow for distinction between different *Enterobacteriaceae* species. Another way of screening is through Polymerase Chain Reaction (PCR)-based technologies, such as GeneXpert® (Cepheid), which has more sensitivity than cultural methods but it is associated with higher costs (29).

In Portugal, the *Direção Geral de Saúde* (DGS) recommends the screening of high-risk patients for CPE carriage on admission, such as patients with recent hospitalization, in dialysis or chemotherapy treatment, who had been colonized or infected by CPE in the past 12 months, or hospitalized in endemic countries. In high-risk areas (ICU, neonatal unit, transplant unit, oncology service) it also recommends that the screening should be repeated on a weekly basis (53).

The decontamination of patients colonized by KPC-producing *K. pneumoniae* has been suggested to reduce transmission and prevent future infections. The antibiotic

regimen to eradicate gut carriage of KPC-producing *K. pneumoniae* uses oral gentamicin or a combination of gentamicin and colistin (50).

The strategy of decolonization has caused some controversy and produced conflicting results (52, 53). Some studies showed that although this therapy can be useful, it also favours secondary resistance to gentamicin and colistin. In that way, this risk should be taken into consideration before starting decolonization and this measure should be reserved for carefully selected patients and special conditions (50, 52).

Since the reservoir for MDR-bacteria colonization is the intestine, approaches to restore a healthy gut microbiota may be proven useful, such as the faecal microbiota transplantation (FMT) (54). FMT consists of the transplantation of the faecal microbiota of a healthy donor into the intestinal tract of a recipient patient. The suspension of stool can be administered via different ways: nasojejunal tube, enemas, colonoscopy or swallowed capsules (55, 56). This strategy has proven to be highly effective against recurrent *Clostridium difficile* infections (55). Recently, there have been some case reports suggesting that FMT resulted in intestinal decolonization of MDR-bacteria, including CPE (54, 57-59).

1.5.3. TREATMENT OF CPE INFECTIONS

Currently, the most common therapeutic options to treat CRE infections include polymyxins, tigecycline, carbapenems, aminoglycosides and ceftazidime-avibactam (29, 60).

Polymyxins (colistin and polymyxin B) were discovered more than 50 years ago and were used for the treatment of Gram-negative bacterial infections. Because of their major adverse effects (neurotoxicity and nephrotoxicity), their use began to decline in the 1980s and they were replaced by the potentially less toxic aminoglycosides. However, in the past few years, due to the increasing resistance of Gram-negative bacteria and to a paucity of new antibiotics, colistin has been brought back to widespread use for the treatment of MDR-organisms, including CPE (29, 50).

Till very recent, resistance to colistin had been linked to mutations in chromosomal genes, and its spread required a *de novo* emergence or a clonal expansion of the resistant isolates. However, a plasmid-mediated colistin resistance gene, *mcr-1*, was reported in *E. coli* from China in 2015 (12) and since then in several *Enterobacteriaceae* species (e.g. *K. pneumoniae*, *E. aerogenes* and *E. cloacae*) in several countries, including in Portugal (61-63). Although *mcr-1* is the predominant one, 7 other variants have been described to date (*mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, *mcr-6*, *mcr-7* and *mcr-8*) (64). The appearance of transmissible colistin resistance poses a significant public health threat, as colistin represents one of the

few available antibiotics against multidrug resistant Gram-negative organisms (63, 65). Tigecycline was designed to circumvent efflux-mediated resistance mechanisms and has very broad spectrum of activity against bacteria, including CPE. Due to its large volume of distribution, tigecycline reaches low concentrations in serum, urine and epithelial lining fluid of the lungs, thus it is not approved for the treatment of patients with bacteraemia, severe pneumonia and urinary tract infection. Although tigecycline remains active against the majority of CPE isolates *in vitro*, resistance is increasing, particularly among patients who had been treated before with this agent. Therefore, tigecycline is mostly used as part of a combination therapy (29, 50, 66).

Paradoxically, carbapenems can be used in the treatment of CPE infections. Several studies showed that prolonged infusion of high-dose carbapenem may have a bactericidal effect when carbapenem MICs of CPE isolates are low (up to 4mg/L). However, treatment with a carbapenem monotherapy is often discouraged (29). On the contrary, dual carbapenem regimens have shown enhanced efficacy over monotherapy and may represent a promising option for infections caused by KPC-producing *K. pneumoniae* isolates. That occurs because the KPC enzyme may have increased affinity for ertapenem, who would act as a decoy, allowing the second carbapenem to exert its activity (29, 66).

Gentamicin and amikacin belong to the class of aminoglycosides. Their side effects, particularly nephrotoxicity, contributed to aminoglycosides lack of use but the scarcity of treatment options has revived the interest of this drug class (60, 67). The use of these drugs in monotherapy has been discouraged due to their lower efficacy relatively to other antibiotics such as β -lactams or due to its toxicity. However, they are important components when used in combination therapy, often with colistin, a carbapenem or tigecycline (29, 68).

Avibactam is a synthetic non β -lactam, β -lactamase inhibitor active against Ambler class A and C β -lactamases and some Ambler class D enzymes, but not MBLs (Ambler class B) (29). The addition of avibactam greatly enhances the activity of ceftazidime against most species of *Enterobacteriaceae* (69, 70). Thus, the combination ceftazidime-avibactam has been successfully used in the treatment of complicated intra-abdominal and urinary tract infections caused by CPE and other Gram-negative bacilli. However, resistant strains of KPC-3-producing-*K. pneumoniae* have already been reported in the last few years (70, 71).

1.6. DETECTION OF CARBAPENEMASES

The rapid identification of CPE is important to ensure early beginning of therapy, as well as the prompt implementation of infection control measures, which is associated with a decreased length of stay, lower mortality and reduced costs (52).

Once carbapenem resistance is suspected through antibiotic susceptibility testing results obtained by disk diffusion or an automated system, additional tests can be used to identify carbapenemase producers. There are several approaches to identify the presence of carbapenemases in *Enterobacteriaceae*, which include molecular, phenotypic and biochemical methods (29, 42).

Non-molecular tests include the modified Hodge test, the carbapenem inactivation method, the Carba NP test or its derivatives and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF MS). All these options target carbapenemase production but none of them are capable of providing the specific carbapenemase type (72).

The Modified Hodge Test is a simple, culture based method that detects the release of carbapenemases into agar media (figure 12). A disk of imipenem is placed on a Mueller-Hinton plate inoculated with a susceptible strain of *E. coli* and then the suspected strain is streaked from the edge of the disk to the periphery of the plate to form a straight line of thick inoculum. If a carbapenemase is produced the imipenem is hydrolysed allowing the susceptible *E. coli* to grow towards the disk, which results in a cloverleaf-like appearance. However, the interpretation of results may be subjective and the sensitivity in detecting MBL-strains, especially NDM, is suboptimal (29, 42, 72). Despite these limitations, this method has good sensitivity and specificity in detecting KPC-producing isolates, so it continues to be included as an acceptable method to detect carbapenemase production (48).

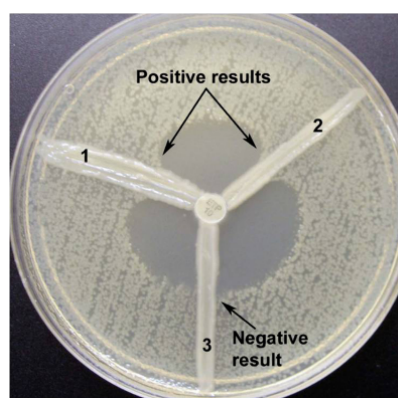


Figure 12 - Modified Hodge test. Adapted from (73)

The carbapenem inactivation method (CIM) is a phenotypic test firstly described in 2015. It uses a meropenem disk that is incubated in a suspension of the test strain and then transferred to a Mueller Hinton plate inoculated with a meropenem susceptible *E. coli* (figure 13). If a carbapenemase is produced, there will be no zone of inhibition around the meropenem disk; in contrast, if no carbapenemase is produced, the meropenem disk will retain its activity and a clear inhibition zone is formed. It is a low cost method, with both sensitivity and specificity reaching 99% for detection of carbapenemases in *Enterobacteriaceae* strains (42, 74).

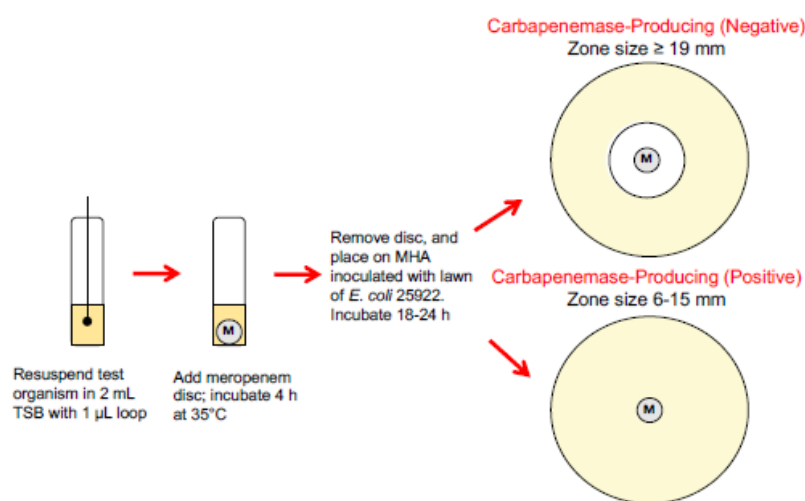


Figure 13 – Overview of carbapenem inactivation method. Adapted from (75)

The Carba NP is a colorimetric biochemical test based on the detection of pH changes caused by *in vitro* hydrolysis of imipenem by carbapenemases. It is highly sensitive in detecting most carbapenemases, except for OXA-48-like and other weak carbapenemases such as for example GES-5 (42, 72). A variant from this test was afterwards proposed that by using a different pH indicator (bromothymol blue) allowed to reduce the cost per reaction, simplify the protocol by the direct use of bacterial cultures and detect OXA-type carbapenemases. It showed a high specificity (100%) and sensitivity (100%) for *Enterobacteriaceae*, *Pseudomonas* spp. and *Acinetobacter* spp. harbouring carbapenemases (76). A commercial version of this test, Rapid Carb Blue kit (Rosco Diagnostica) is currently available, using tablets containing imipenem and a negative control (77).

MALDI-TOF MS (figure 14) is a rapid and reliable method for microbial identification at a species level by comparing the peptide mass fingerprint (PMF) pattern with a database containing reference spectra (78, 79). It is based on the identification of the mass-to-charge (m/z) ratio of the proteins within the clinical sample. These m/z ratios are generated mostly

from ribosomal proteins, which are highly abundant and unique to bacterial groups or species (78, 80). Detection of carbapenemases is based on the identification of a peak resulting from the hydrolysis of the carbapenem after incubation of the test strain in a carbapenem solution. It has shown high sensitivity (96,7%) and specificity (97,8%) (81).

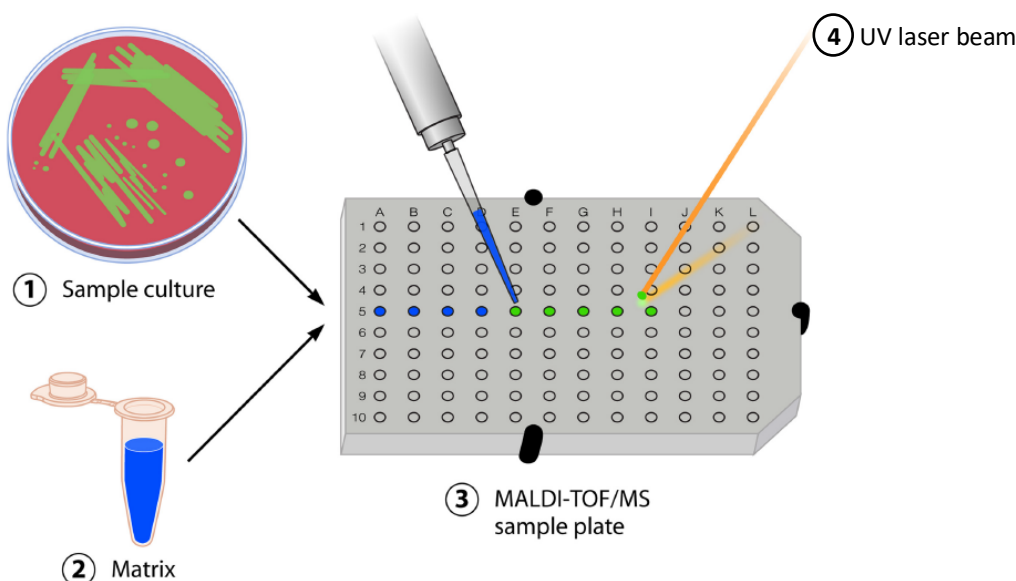


Figure 14 - Schematic for bacterial identification by MALDI-TOF MS. Adapted from (78)

Molecular assays for detection of carbapenemase producers include PCR, microarrays and whole-genome sequencing (WGS) (42, 72). Their advantages include high sensitivity and specific genetic information (48). However, with these molecular approaches only known genes can be targeted, so those encoding novel carbapenemase will be missed (72). Also, molecular assays are the most expensive method for carbapenemase production detection, due to the high costs of reagents and equipment, and required experienced personnel in molecular biology (48).

PCR allows for rapid identification of specific carbapenemases genes, either by conventional simplex or multiplex PCR. PCR-based commercial assays, such as Xpert Carba-R (Cepheid) have also been proposed (42, 72). This system is a real time PCR assay that detects KPC, NDM, VIM, IMP and OXA-48 from rectal swab specimens or pure colonies, with a sensitivity between 97% and 100% (72, 82). It also allows a rapid identification of carbapenemase-producing organisms, with a turnaround time of approximately one hour (82). Nevertheless, Xpert Carba-R only detects the presence of carbapenemase genes, providing no information regarding the species carrying them (42).

Microarray technology can be combined with PCR amplification of target sequences or can be used directly in DNA from bacterial isolates or patients specimens. This method can include higher number of targets than PCR assays and is also capable of distinguish between closely related variants (72). Numerous microarray systems have been developed, including Verigene (Luminex Corporation), which can identify the genus and species of multiple Gram-negative organisms, as well as the presence of several resistance markers (KPC, NDM, CTX-M, VIM, IMP and OXA), directly from positive blood cultures (83). It has an approximate turnaround time of 2 hours and a high sensitivity (about 97%) (84-86). On the other hand, it is a method with substantial material costs (approximately 50-70 euros per test) (85).

Finally, whole-genome sequencing (WGS) can query the entire bacterial genome for all known antibiotic resistant mechanisms, not only carbapenemases (42, 72). Furthermore, it has the ability to provide information on the type of plasmids carrying resistance genes and also on the relatedness of isolates (72). Presently, WGS is still an expensive technology but its application in clinical microbiology laboratories is in sight (42, 72).

CHAPTER 2 - OBJECTIVES OF THE STUDY

2.1. STATEMENT OF OBJECTIVES

Carbapenems are β -lactam antibiotics considered the treatment of choice for infections caused by MDR-*Enterobacteriaceae*. However, carbapenemase-producing *Enterobacteriaceae* (CPE) are increasingly reported worldwide, compromising the effectiveness of these drugs and a return to a pre-antibiotic era. Their rapid dissemination is mainly due to the acquisition of genes located on MGEs (integrons, transposons, plasmids) encoding a high diversity of carbapenemases. These enzymes have been reported all over the world, with significant geographical differences in the prevalence and epidemiology, being those of the KPC-type the most widely distributed, including in Portugal.

In our country, the incidence of CPE in healthcare institutions is increasing and outbreaks caused by KPC-3-producing *Enterobacteriaceae* have been described. In this context, strict infection control measures for infected patients and active screening of colonized patients are being implemented in different Portuguese hospitals to prevent the spread of these organisms, apparently with a low success. However, the reasons for which their application was not successful in decreasing transmission of CRE strains are poorly understood.

The **general aim** of the present study was to understand the dynamics of CPE transmission among infected or colonized patients that were hospitalized in one hospital in the North of Portugal (November 2017- February 2018), in order to contribute to improve preventive infection control decisions. To accomplish this aim, molecular characterization of the isolates was performed considering the following **specific aims**:

1. To evaluate the occurrence and diversity of CPE in the different units of the hospital, including isolates colonizing and infecting patients, and establish CPE evolution throughout time.
2. To analyse epidemiological data of isolates colonizing or infecting patients from the same healthcare institution to assess the risk of developing infection.
3. To propose CPE transmission routes by relating molecular, epidemiological and clinical information available, that would allow identifying at-risk units or patients.

CHAPTER 3 - MATERIALS AND METHODS

The present study focused on CPE isolates collected from colonized or infected patients. A schematic view of the experimental work developed for the characterization of these isolates is shown in figure 20.

3.1. COLLECTION OF SAMPLES AND SPECIES IDENTIFICATION

A total of one hundred and thirty-two presumptive CPE isolates recovered from hospitalized patients (n = 132) at a hospital from the north of Portugal between November 2017 and February 2018 were collected. They included 97 isolates recovered from faecal samples (February 2018) and 35 isolates recovered from clinical specimens (November 2017 to February 2018).

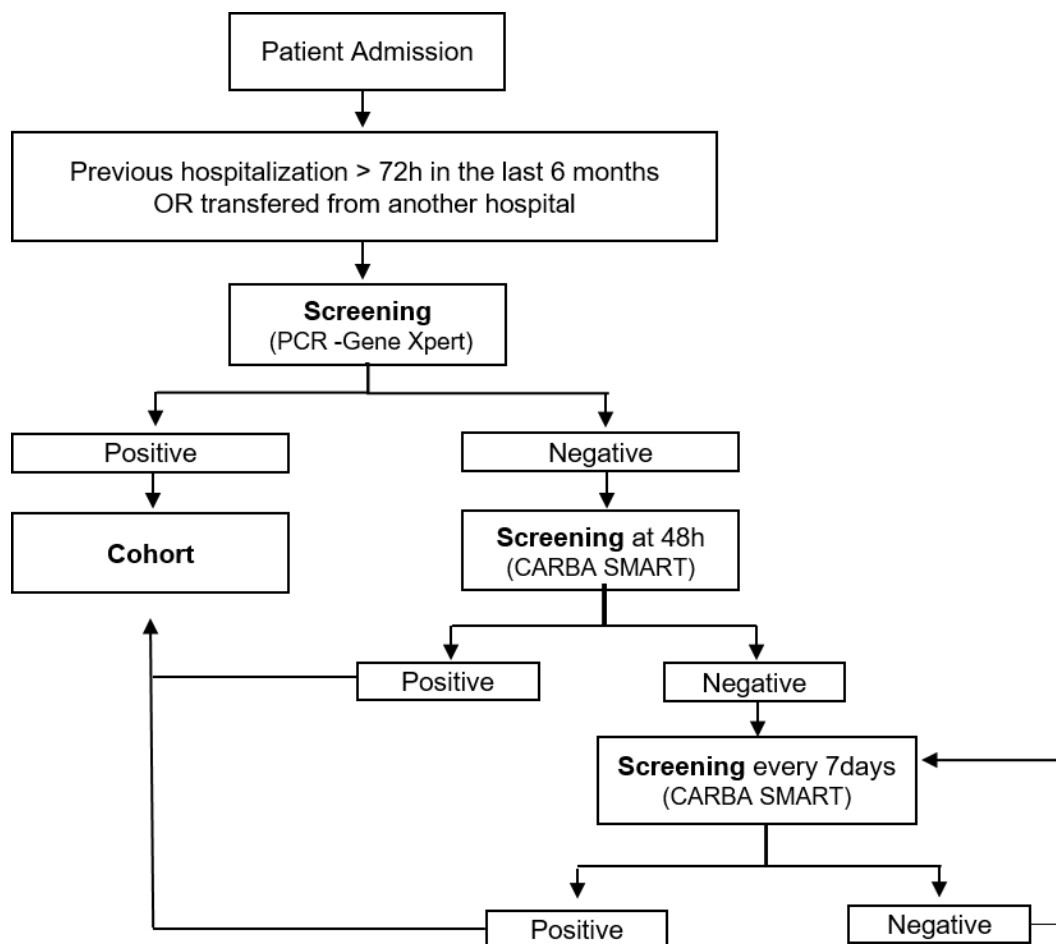


Figure 15 - Screening protocol adopted by the hospital.

The CPE screenings at this hospital were performed at admission, according to the protocol adopted (figure 15), through a molecular biology technique (PCR, GeneXpert) and

subsequent colonization was confirmed periodically by a cultural method (chromogenic selective medium, chromID Carba Smart from bioMérieux) (figure 16).

The screenings at admission were performed at high-risk patients, that is, with a history of hospitalization in the last 6 months and for a period of time exceeding 72 hours, or patients that were transferred from another healthcare institution. Screenings by inoculation on chromogenic agar were performed after 48h of hospitalization in patients with negative results at admission and every 7 days in hospitalized patients. All patients with positive results were transferred to cohort areas and submitted to infection control measures (hand hygiene, use of personal protective equipment, restriction of visiting hours and reinforcement of environmental control measures).

Identification of clinical isolates and antibiotic susceptibility testing were performed by VITEK (bioMérieux, Marcy l'Etoile, France) at the microbiology laboratory from the hospital. CPE isolates from rectal swabs of colonized patients (i.e. asymptomatic carriage of CPE isolates on the gastrointestinal tract) were presumptively identified by inoculation and growth in selective and chromogenic screening commercial media (chromID CARBA-SMART, bioMérieux) after 18h of incubation at 37°C. Only colonies with the appearance of *E. coli* (pink to burgundy colonies) or KESC - *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter* (bluish-green colonies) were selected. Isolates identification was further confirmed by MALDI-TOF MS and species-specific PCR (87). Non-*Enterobacteriaceae* isolates were discarded.

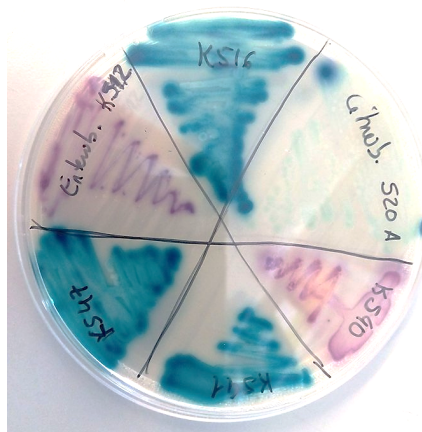


Figure 16 - Isolates on chromogenic medium. K547, K516, K541: *K. pneumoniae*; K540: *E. coli*; K512: *Enterobacter* sp; K520A: *Citrobacter* sp.

For all patients, demographic data including age, sex, pathologies and previous hospitalization data, as well as date of sample collection, was recorded.

3.2. DETECTION AND IDENTIFICATION OF CARBAPENEMASES

Carbapenemase production was initially assessed at the hospital for 29% of the clinical isolates by the immunochromatographic assay Letitest® (LetiDiagnosis Barcelona, Spain) or real-time PCR Xpert® Carba-R (Sunnyvale, USA, Cepheid). The presence of carbapenemases was further screened for all isolates by the Blue-Carba test (76) and confirmed by multiplex PCR (88).

The bacterial strains used for Blue-Carba test were obtained from Mueller-Hinton agar. A loop of the bacterial culture was added to both test solution (prepared with Tienam® 500 -equivalent to 3mg of imipenem monohydrate-, bromothymol blue at pH 6 and zinc sulphate 10mM) and to the negative control solution (bromothymol blue at pH 7) in a 96-well flat bottom plate. The results were read after 2 hours of incubation, although some samples had a positive result in a few minutes. According to the protocol, a positive result is obtained when there's a difference in colour between the test solution and the negative control, for example: yellow/blue, yellow/green, green/blue (figure 17).

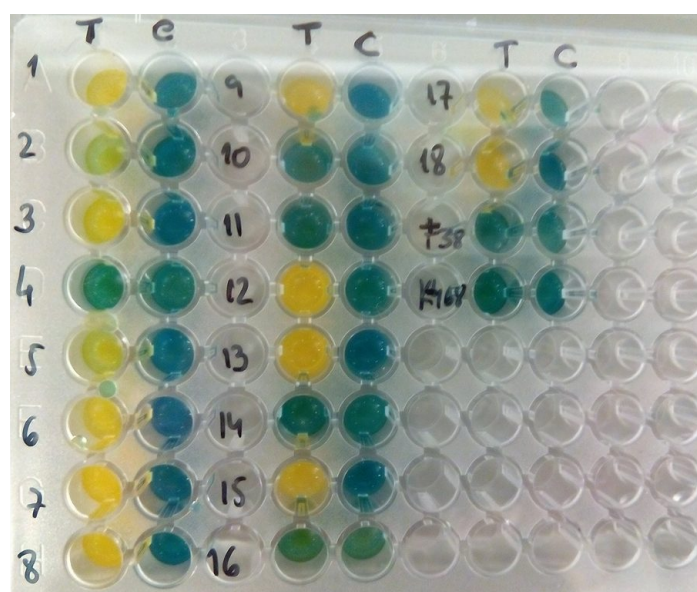


Figure 17 - An example of a Blue-Carba test.

The presence and type of carbapenemase was confirmed by PCR directed to the most frequent carbapenemase gene families (*bla*_{KPC}, *bla*_{OXA-48}, *bla*_{IMP}, *bla*_{VIM}, *bla*_{NDM}) (88) and further sequencing of amplicons obtained from representative isolates.

3.3. POPULATION STRUCTURE OF CARBAPENEMASE-PRODUCING *K. PNEUMONIAE* ISOLATES

The relationship among *K. pneumoniae* isolates was inferred by Fourier transform infrared (FTIR) spectroscopy, a technique that enables the rapid and non-destructive discrimination of bacterial isolates, providing a fingerprint spectra of the microbial strains, that reflects a specific fingerprint of the biochemical composition of the bacterial cell, that in *K. pneumoniae* has been correlated with the capsular type (K-type) composition.

The isolates were grown on Mueller-Hinton agar at 37°C during 18h and then applied directly on the attenuated total reflectance (ATR) crystal since the FTIR was used in the ATR mode. Spectra were acquired at 4000-600 cm⁻¹ with 4 cm⁻¹ resolution and 32 scan co-additions. Spectral data was further treated and interpreted by multivariate data analysis using Matlab R2015a version 8.5 (MathWorks, Natick, MA) and PLS Toolbox version 8.5 for Matlab (Eigenvector Research, Manson, WA, USA). The isolates were grouped according to their spectra similarity and were compared with those included in an in-house database that includes representative *K. pneumoniae* isolates from main multidrug resistant clones and capsular types circulating in healthcare settings in our geographic region and worldwide. This comparison allowed K-type prediction that was subsequently confirmed for representative isolates by sequencing of the *wzi* gene (89). The *wzi* gene codes for the outer membrane Wzi, involved in capsule attachment to cell surface, which can be used as a method for K-type prediction. It is one of the six conserved genes (*galF*, *orf2*, *wzi*, *wza*, *wzb* and *wzc*) present in the genomic locus responsible for capsular type production. It is known that its allelic variability can be correlated with K-type (<http://bigsdbs.pasteur.fr>) (89).

3.4. POPULATION STRUCTURE OF CARBAPENEMASE PRODUCING *E. COLI* ISOLATES

Identification of *E. coli* phylogenetic groups was performed by PCR (figure 18), following the quadriplex method described by Clermont *et al.* (90), where *E. coli* isolates are assigned into eight phylo-groups (A, B1, B2, C, D, E and clade I). The identification of the phylo-group is based on the presence or absence of four genes (*arpA*, *chuA*, *yjA* and TspE4.C2), which may result in one of fifteen different genotypes. The distinction between some phylo-groups (A/C, D/E, E/clade I) requires additional testing with specific primers (90).

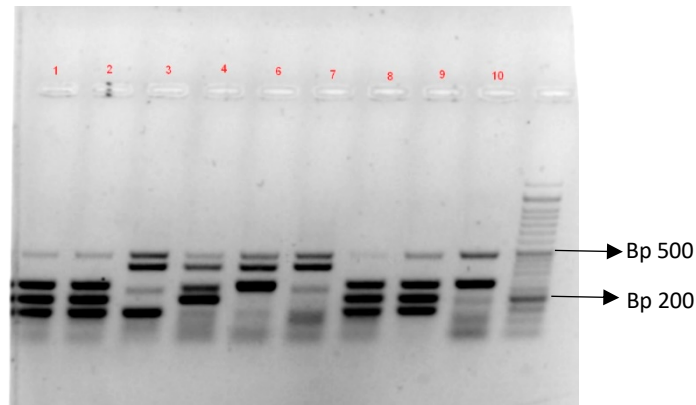


Figure 18 - PCR profiles of *E. coli* strains.

Considering the high occurrence of B2-ST131 *E. coli* in Portuguese clinical settings producing ESBL, and its high relevance as a clinical pathogen or human colonizer (91) the presence of *E. coli* B2-ST131 was screened by PCR among *E. coli* isolates belonging to the phylogenetic group B2 (92).

3.5. MOLECULAR TECHNIQUES

3.5.1. DNA EXTRACTION

DNA templates were prepared by suspending a freshly grown colony in 20 µL of NaOH + SDS and heating at 100 °C for 15 minutes. After adding 180 µL of ultrapure sterile water, the extracts were submitted to microcentrifugation at 13000 rpm for 15 minutes. The supernatants were then stored at -4°C until use.

3.5.2. DNA AMPLIFICATION

PCR is a molecular biology technique that allows the rapid amplification of desired sequences of DNA, generating a large amount of copies (93). PCR is based on the ability of DNA polymerase to assemble new strand of DNA from the existing DNA template and nucleotides (94). Thus, this technique requires six basic components (95-97):

- template DNA that contains the target sequence to be amplified;
- a pair of oligonucleotides (forward and reverse) that flank the target sequence;
- di-deoxynucleotide triphosphates (dNTPs);
- a thermostable *Taq* DNA polymerase;
- Mg²⁺ ions (cofactor of the enzyme);
- buffer solution, which supplies the correct pH for the activity of the enzyme.

The above mentioned components are mixed together and taken through series of three cyclic steps that require different temperature conditions, conducted on a thermocycler. In the first step (denaturation), temperature is increased to approximately 94°C to separate the double strand of DNA. In the second step, the reaction temperature is rapidly lowered (55°C-65°C) to allow the primers to bind to the complementary strands of DNA, a process known as annealing. In the third step, the temperature is raised again (72°C) to allow DNA polymerase to add nucleotides to synthesise new DNA strands, in a process called elongation. Each repetition of these three steps doubles the number of copied DNA molecules (copy number 2^n , where n is the number of cycles), resulting in an exponential amplification (95, 96).

The visualization of the PCR product is usually done by agarose gel electrophoresis, where products are separated based on size using DNA intercalating agents for staining (96). A predetermined set of DNA products with known size may be running simultaneously on agarose gel, along with a molecular weight marker to help determine the size of DNA fragments (95).

For this study, PCR reaction mixture was prepared using NZYTaq II 2× Green Master Mix (nzytech, Portugal), which contains a new generation of *Taq*-derived DNA polymerases optimized for standard PCR applications (NZYTaq II DNA polymerase). This master mix also contains dNTPs, buffer and additives.

PCR products were directly loaded on 1.5%-2% agarose gel prepared with TAE 1X and containing SYBR® Safe DNA gel stain. A molecular weight marker, NZYDNA Ladder VI (nzytech, Portugal) ran simultaneously with the DNA fragments, at 90V during 40 minutes. After electrophoresis, gels were read and photographed under UV light, using the Gel Doc XR+ with Image Lab Software (Biorad, EUA).

3.5.3. DNA PURIFICATION

The presence of impurities or/and excess oligonucleotide primers can be problematic when sequencing direct PCR products. Therefore, the selected PCR products (KPC and *wzi* products) were purified with the kit NZYGelpure (nzytech, Portugal) according to the manufacturer's instructions (figure 19).

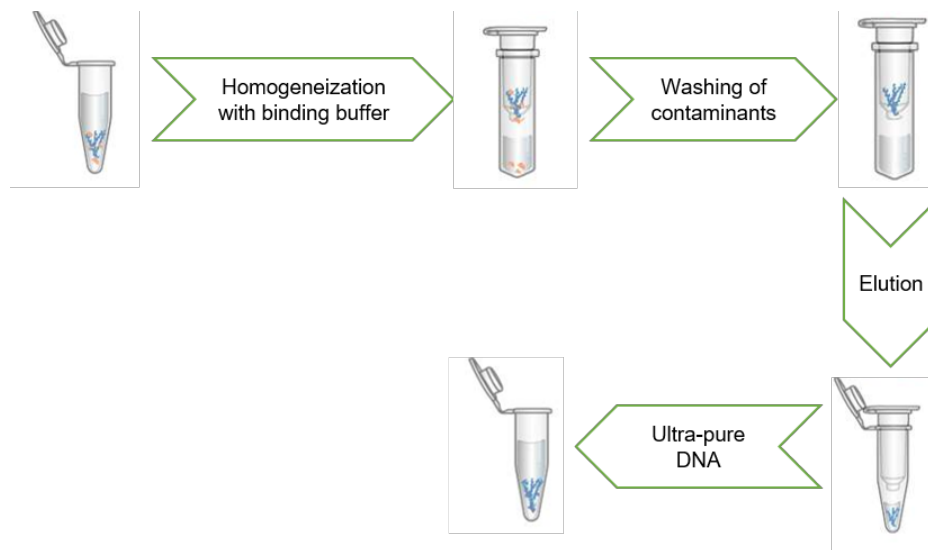


Figure 19 - An overview of the DNA purification method by nzytech.

This kit uses a silica-gel based membrane that selectively adsorbs the DNA fragments, allowing the impurities to be washed away. The DNA fragments are then eluted off the column to a new test tube, obtaining an ultra-pure DNA that can be used for sequencing.

3.5.4. DNA SEQUENCING

The purified PCR products were sequenced by the outsourcing services of GATC Biotech (Konstanz, Germany).

The DNA sequences were analysed and compared with those at the NCBI database, as well as with those at Institute Pasteur MLST database (<http://bigsd.b.pasteur.fr/klebsiella/>), using Chromas software (Technelysum, Australia), available online.

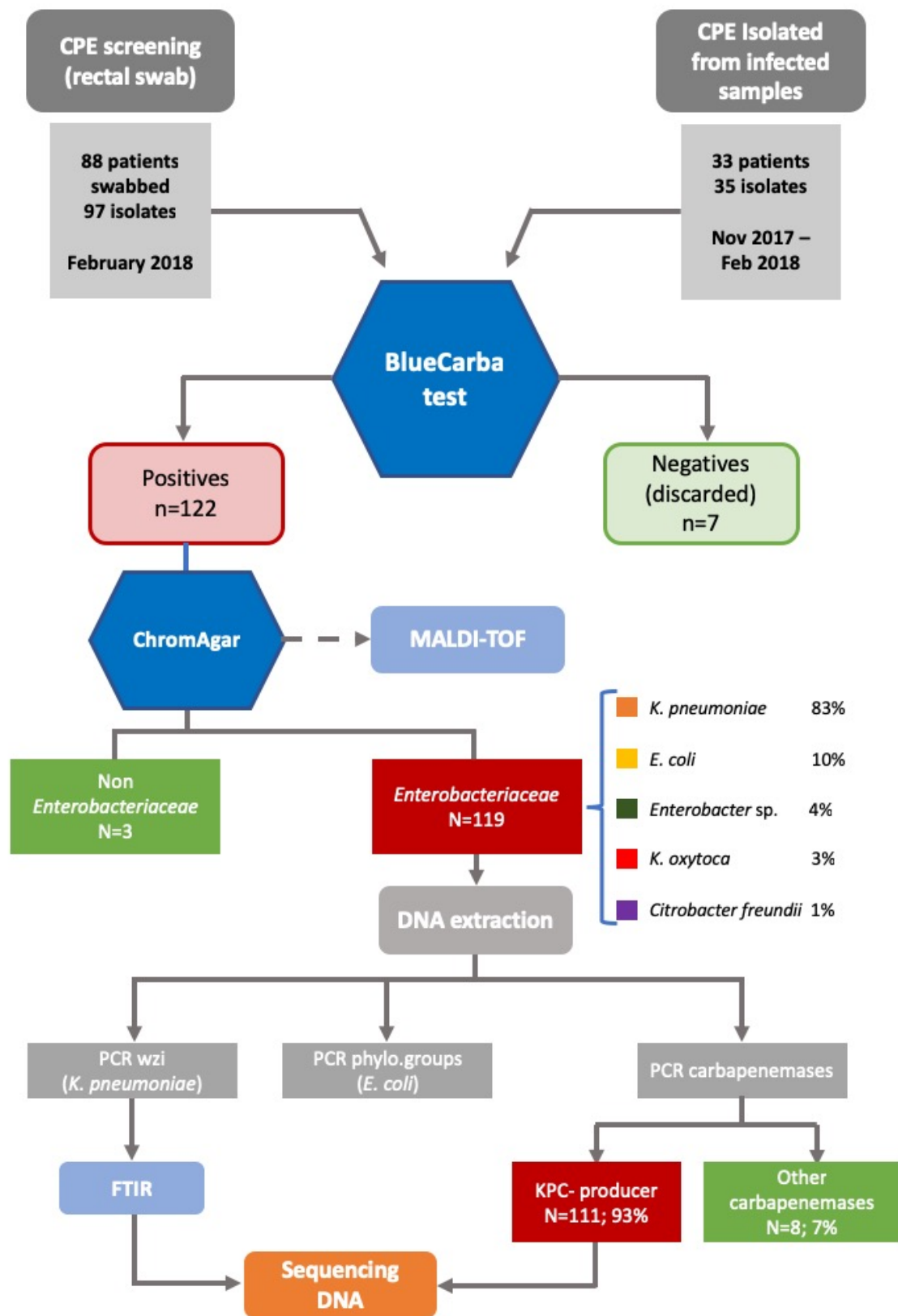


Figure 20 - A schematic view of the experimental work.

CHAPTER 4 - RESULTS AND DISCUSSION

4.1. EPIDEMIOLOGY OF BACTERIAL ISOLATES

From the 132 presumptive CPE initially collected from clinical and faecal samples, 122 were positive for carbapenemase production (92%). Ten isolates turned to be Blue-Carba negative (non-carbapenemase producers) and may have grown on selective media due to decreased susceptibility to carbapenems caused by a combination of porin permeability and production of ESBLs. From those 122 isolates, three faecal isolates didn't belong to *Enterobacteriaceae* (*Aeromonas hydrophila*, *Enterococcus faecium* and *A. baumannii*). These isolates were discarded since they were not the target of this study.

One hundred and nineteen CPE isolates recovered from a total of 114 patients constituted our final sample and were further characterized (n=85 from faecal samples between November 2017 and February 2018, n=34 from clinical samples from February 2018).

Patient's age ranged from 20 years to 96 years, with mean age of 74 ± 14 years (median 78 years). A total of 67 (59%) CPE isolates were from female patients whereas 46 isolates were from males, corresponding to a male female ratio of 1:1,43. The prevalence was higher on women in both colonization and infection samples (table 3).

Table 3 - Gender distribution of patients with CPE.

Gender	Colonization no. (%)	Infection no. (%)	Total no. (%)
Female	48 (60%)	19 (58%)	67 (59%)
Male	32 (40%)	14 (42%)	46 (41%)
Total	80 (100%)	33 (100%)	113 (100%)

Note: One female patient had both a colonizing and an infecting strain.

Clinical isolates were obtained from urine (n=20; 57%), pus (n=8; 23%), blood culture (n=3; 9%), sputum (n=1; 3%), tracheal aspirate (n=1; 3%) and one liquid from wound debridement (n=1; 3%). The large number of urine samples highlights the important role of these enterobacterial species (namely *K. pneumoniae* and *E. coli*) in urinary tract infections, particularly in an elderly population.

K. pneumoniae was the most common *Enterobacteriaceae* isolated (83%) from both colonization (n=66/85, 78%) and infection (n=32/34, 94%) samples, followed by *E. coli* (9%) and *E. cloacae* complex (4%), both mostly from colonization samples (table 4). While nearly all isolates from infection belonged to *K. pneumoniae* (94%), the isolates from faecal samples presented a greater diversity, including mostly *K. pneumoniae* (78%), but also *E. coli* (11%), *E. cloacae* complex (5%), *K. oxytoca* (5%) and *Citrobacter freundii* complex (1%), the latter two species appearing exclusively in colonization samples.

Table 4 - Distribution of CPE isolates among clinical and faecal specimens.

Microorganism	Colonization		Infection		Total	
	No.	%	No.	%	No.	%
<i>Klebsiella pneumoniae</i>	66	78%	32	94%	98	83%
<i>Escherichia coli</i>	10	11%	1	3%	11	10%
<i>Enterobacter cloacae</i> complex	4	5%	1	3%	5	4%
<i>Klebsiella oxytoca</i>	4	5%	0	0	4	3%
<i>Citrobacter freundii</i> complex	1	1%	0	0	1	1%
Total	85	100%	34	100%	119	100%

Within the study population, one patient (female, 81 years old) had both an infecting and a colonizing strain identified on 12th and 15th of February 2018, respectively (figure 21). The patient had been hospitalized due to a respiratory tract infection caused by respiratory syncytial virus (RSV). The first CPE screening was performed three days after admission and turned out negative. During hospitalization, the patient acquired a urinary tract infection and a carbapenem-resistant *K. pneumoniae* was isolated from the urine sample collected for analysis. A new screening for CPE by culture of a rectal swab was performed three days later, with a positive result. Isolates from both faecal and urine samples was identified as *K. pneumoniae* producing KPC-3 and both strains were possibly related since they contained the same *wzi* allele (*wzi*187) and belonged to the same K-type (KL27).

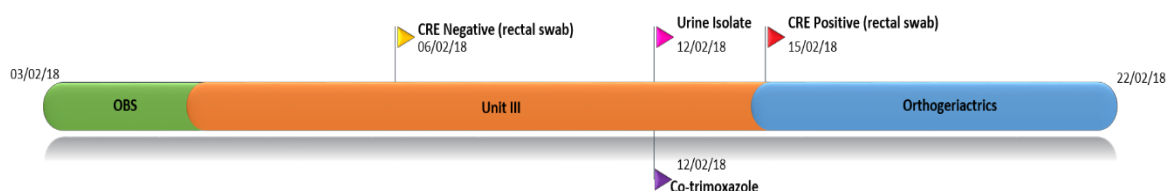


Figure 21 - Timeline describing the evolution of the patient with colonized and infected samples.

Since the infected isolate was reported before the colonized one, we can wonder if CPE colonization was not a prerequisite for infection or if the time between colonization and infection was too short (6 days between the two screenings).

4.2. DIVERSITY OF CPE IN THE DIFFERENT UNITS OF THE HOSPITAL

In an attempt to understand why transmission rates of MDR-bacteria were so high in this hospital, we analysed the distribution of patients with CPE isolates among the different hospital units (figure 22) that were divided in four departments: medical units, surgical units, emergency room and infectious diseases (including only patients selected for decolonization). The fact that both colonization and infection isolates were mostly recovered from medical and surgical units suggests that the most probable origin of the bacteria responsible for infections is previous colonization of patients or dissemination through problematic wards.

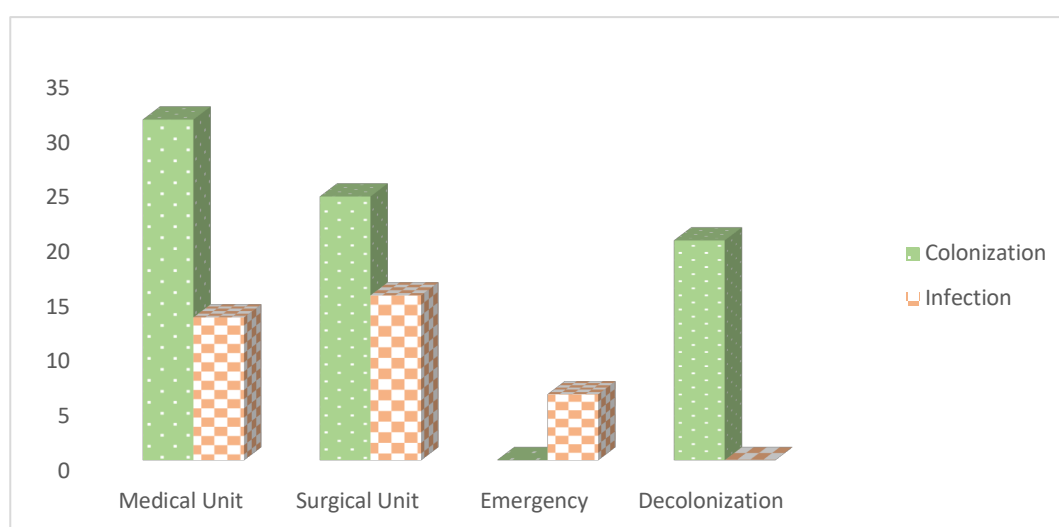


Figure 22 – Distribution of colonized and infected patients among hospital units.

Faecal colonization isolates were collected from patients at medical units (n=38; 45%), surgical units (n= 25, 29%), whereas part of them (n=22, 26%) were isolated from patients that had been selected for decolonization.

From the 20 patients that were doing **decolonization**, 12 were recent CPE carriers (with positive screenings since December 2017). From the 8 patients with older positive screenings, 7 of them (87,5%) had negative CPE screenings at some point during the treatment (figure 23). The time required for these patients to become non-carriers was on average 208 days (6 months). However, all of these patients had subsequent positive screenings and had become CPE carriers again that in two cases (n=2/7; 29%) occurred during a new hospitalization in this hospital. This suggests a transmission from other patients or healthcare staff and therefore a failure in the infection control measures. The

mean time between the two screenings (a positive result following a negative result) was 9 days. The other five patients (71%) had no recent hospitalizations, therefore the cause of the recolonization is not clarified.

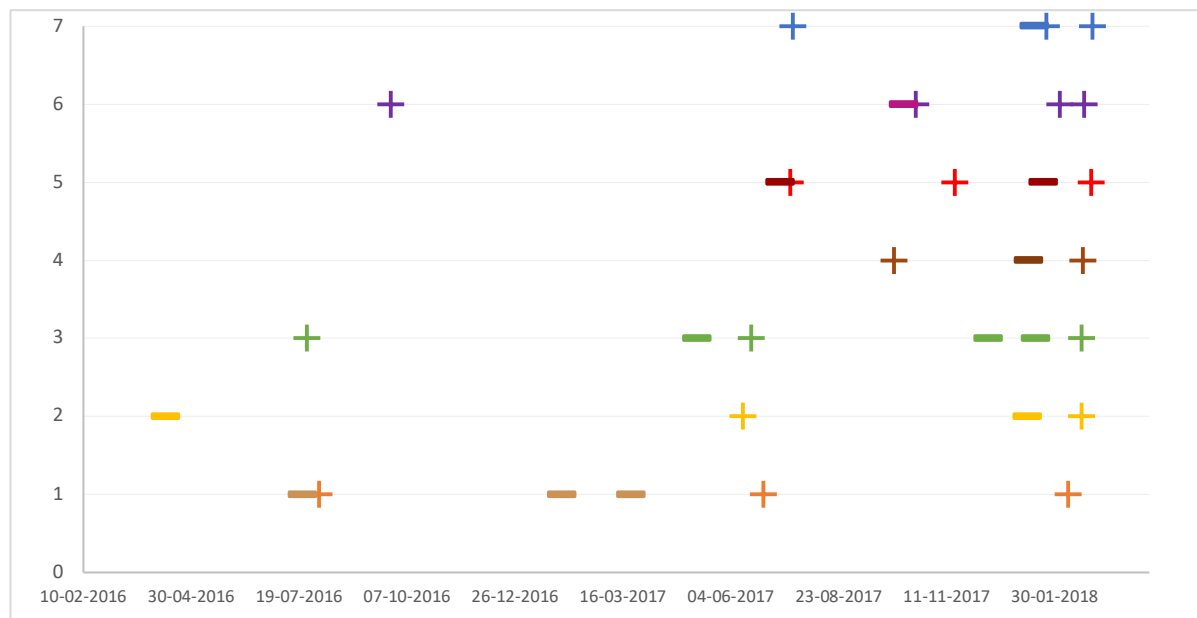


Figure 23 - Evolution of patients during decolonization treatment.

Two species of CPE (*K. pneumoniae* and *E. coli*) were isolated from rectal samples of two different patients. Both were KPC-3 producers, which may indicate the transfer of a KPC-3 encoding-plasmid from one species to the other.

Gut decolonization is a controversial method, with some studies suggesting that it is not only ineffective but also increases the risk of associated - drug resistance (54). In this hospital, the decolonization of patients was performed not by administration of antimicrobials, but by faecal microbiota transplantation. Therefore, the development of drug resistance to colistin or gentamicin (drugs frequently used in decolonization treatments) poses no threat for these patients. In other studies, FMT has shown potential for decolonization of gut MDR-bacteria (54, 57-59). However, in this case, the long time required for the success of the decolonization treatment along with the rapid recolonization that follows seems to indicate that the decontamination of colonized patients by FMT is not an effective measure and may be an economic burden to healthcare systems.

Nonetheless, some aspects of this population should be clarified, such as the criteria used for the selection of these patients by the medical staff and the periodicity of CPE screenings, to conclude more about the effectiveness of this measure.

Within the medical department (figure 24-A), most of colonization isolates came from internal medicine (n=14; 36,8%) and unit III (n=12; 31,6%). Other units were otorhinolaryngology (n=4; 10,5%), pulmonology (n=2; 5,3%), nephrology (n=2; 5,3%), urology (n=1; 2,6%), ophthalmology (n=1; 2,6%), neurology (n=1; 2,6%) and observation

room (n=1; 2,6%). Regarding the surgical units (Figure 24-B), women surgery was the unit with the highest number of isolates (n=5; 20%), followed by plastic (n=4; 16%), men surgery (n=4; 16%) and orthopaedics (n=4; 16%). Other surgical units were neurosurgery (n=3; 12%), vascular surgery (n=3; 12%) and cardiothoracic surgery (n=2; 8%).

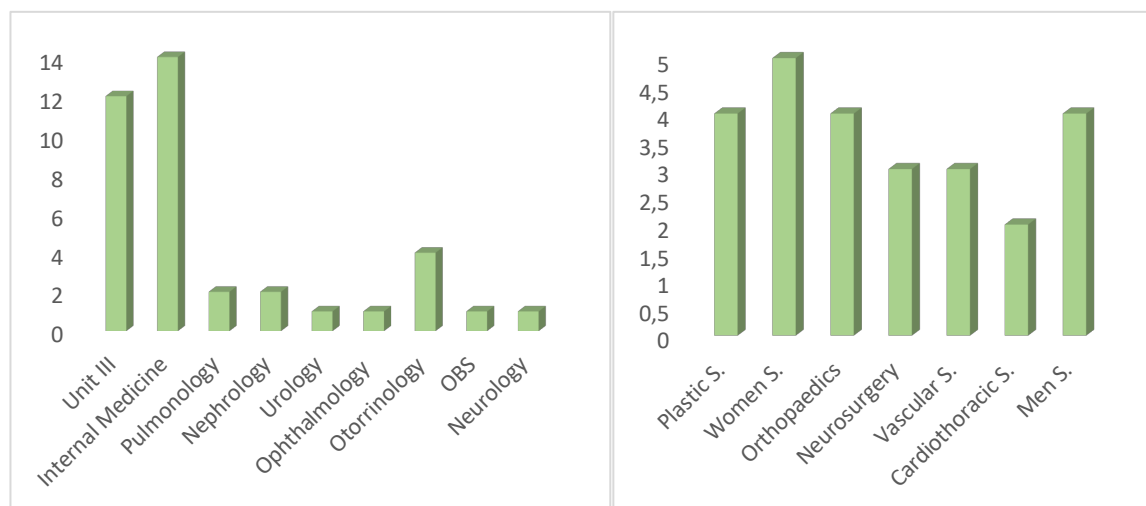


Figure 24 - (A) Distribution of colonization isolates among units of the medical department; **(B)** Distribution of colonization isolates among units at the surgical department.

Clinical isolates were frequently isolated from surgical (n=15; 44%) or medical (n=13; 38%) units and 18% (n=6) were isolated from the emergency room (figure 22).

From the six outpatients (table 5), three were CPE carriers since their last hospitalization. The other three had no history of CPE screenings, but two of them were hospitalized in the last month. This data strongly suggests a nosocomial acquisition of CPE. The enterobacterial species identified in all isolates was *K. pneumoniae*. In all cases but one, *K. pneumoniae* carried the *wzi64* gene and belonged to K-type 64. The CPE isolate was identified in the urine (5 out of 6 patients) and blood samples (2 out of 6 patients).

Table 5 - CPE isolates identified from the emergency room.

Isolate code	Gender	Age	Pathology	Sample origin	Bacterial species	wzi/K-type	Previous screenings	Previous hospitalization
K533 K536	M	74	prostatitis	urine blood	<i>K. pneumoniae</i>	wzi64/K64 KL64	none	unknown
K563	M	79	dehydration	urine	<i>K. pneumoniae</i>	wzi64/K64 KL64	+	4 months
K564	M	87	recurrent UTI	urine	<i>K. pneumoniae</i>	wzi64/K64 KL64	none	1 month
K571	F	94	gastroenteritis	urine	<i>K. pneumoniae</i>	wzi64/K64 KL64	+	8 months
K575	F	79	pielonephritis	blood	<i>K. pneumoniae</i>	unknown	+	6 days
K581	F	79	recurrent UTI	urine	<i>K. pneumoniae</i>	wzi64/K64 KL64	none	1 month

The K533 and K536 isolates belonged to a male patient that was diagnosed with a prostatitis caused by CPE. He first received gentamicin, then a combination of ertapenem, meropenem and colistin and finally ceftazidime plus avibactam. However, he had a poor outcome and eventually died. This highlights the threat that infections by MDR-bacteria represent to patients, being associated with high mortality, especially in cases of bacteremia and in the elderly (6).

Among the clinical isolates from the medical department (figure 25-A), internal medicine was the most prevalent unit (n=7; 54%), followed by urology (n=2; 15%) and unit III (n=2; 15%). One isolate was identified from AVC unit (n=1; 8%). Similarly to faecal samples, women surgery was the unit in the surgical department (figure 25-B) with the highest number of positive screening (n=8; 50%) but in this case it was followed by vascular surgery (n=3; 19%). Other isolates were identified among samples from patients at plastic surgery (n=2; 13%), cardiothoracic (n=1; 6%), orthopaedics (n=1; 6%) and men surgery (n=1; 6%).

The identification of patients with CPE isolates in 9 medical wards and 7 surgical departments shows that CPE are spread among several units in the hospital. The fact that the internal medicine unit alone harboured 42% (n=21/50) of the cases from the medical department and that the women surgery unit alone harboured 30% (n=12/40) of the cases from the surgical department suggests a lower adherence to infection control measures in these units by the healthcare personnel. Also, the possibility of cross contamination due to patient's and staff's mobility or even medical equipment and surface's contamination should be considered.

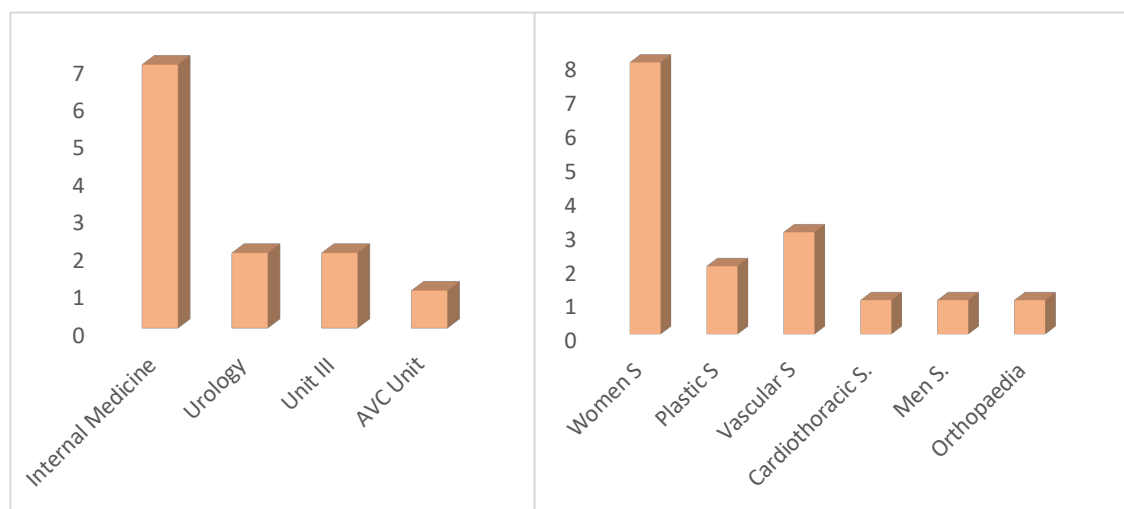


Figure 25 - (A) Distribution of infection isolates among units of the medical department; (B) Distribution of infection isolates among units at the surgical department.

In other studies that took place in hospitals, including in Portugal (35, 98), units like emergency room, surgery wards and internal medicine have shown the highest incidence of CPE, similarly to what is seen in this study. This can be explained by the high number of invasive procedures, elderly patients and long hospitalization periods in these units.

Regarding the microbiological profile of the faecal isolates among the several units (figure 26), *K. pneumoniae* was more frequently isolated from medical units (n=33/66; 50%) but it was also highly found in surgical units (n=18/66; 27%) and decolonization patients (n=15/66; 23%). *E. coli* isolates were mostly recovered from patients undergoing decolonization protocol (n=6/10; 60%), followed by medical units (n=3/10; 30%). *E. cloacae complex* was mainly found in patients from surgical units (n=3/4; 75%), particularly women and plastic surgery. *K. oxytoca* (n=2/4; 50%) and *C. freundii complex* (n=1) were also present in surgical units.

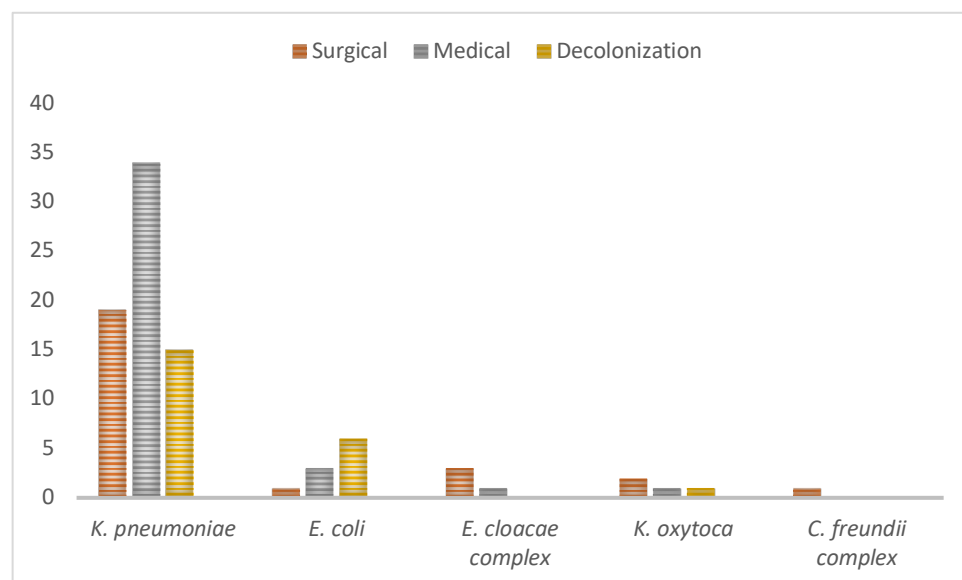


Figure 26 – Distribution of faecal samples by species among hospital units.

Regarding clinical samples (figure 27), the pathogens found in surgical sites infections were *K. pneumoniae* (n=14/32; 44%) and *E. cloacae complex* (n=1/1; 100%). The only *E. coli* isolate from clinical samples was recovered from a medical unit (n=1/1; 100%).

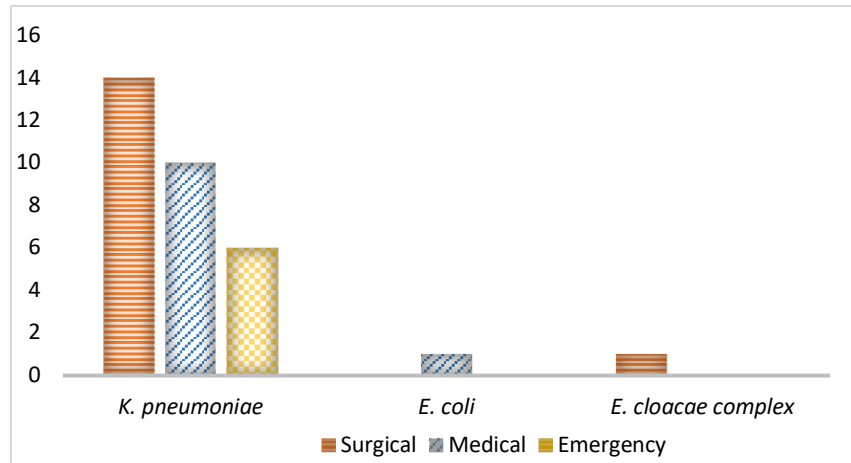


Figure 27 - Distribution of infection samples by species among hospital units.

4.3. RISK FACTORS FOR CPE COLONIZATION

A high proportion of patients became colonized by CPE during their current (n=41; 53%) or previous (n=21; 26%) hospitalization in this hospital. For the remaining patients (n=18; 21%), the carrier status before this hospitalization was not clearly known. Nevertheless, it can be concluded that nearly all patients (n=62; 78%) became colonized by CPE after their admission at this hospital, so CPE colonization seems to be largely nosocomial.

From the 80 different colonized patients, 41 (51%) of them had *de novo* CPE colonization while 26 patients (33%) had previous positive screenings. The remaining 13 patients (16%) had no history of screenings. The *de novo* patients were hospitalized mostly in unit III, internal medicine and surgical wards. Most of the patients with previous positive screenings (20/26; 77%) were under decolonization protocols.

Among the 41 *de novo* colonized patients, 27 (66%) had a long **hospitalization period** (>14 days) which seems to be an important risk factor for CPE colonization in this healthcare institution. This factor has been reported in many studies to contribute to CPE colonization (12, 50, 99). From the 14 patients under short hospitalization period (<14 days), 12 had antibiotherapy treatments (amoxicillin plus clavulanic acid, piperacillin plus tazobactam, 3rd generation cephalosporins, levofloxacin, trimethoprim/sulfamethoxazole, azithromycin and gentamicin). The other 2 patients with short hospitalization period had no antibiotic exposure and both were on surgical units. These patients may have been exposed to other risk factors, like invasive procedures or catheters, but such information was not initially collected.

In relation to **antibiotic exposure**, 38 from the 41 *de novo* colonized patients (93%) were exposed to antimicrobials in the previous three months (table 6). The main antibiotics

administered were piperacillin plus tazobactam (n=19; 26%), amoxicillin plus clavulanic acid (n=14; 19%), ceftriaxone (n=7; 10%) and vancomycin (n=5; 7%). Antibiotic exposure in the previous three months was significantly common among CPE carriers, particularly the administration of β -lactams/ β -lactamase inhibitors (piperacillin plus tazobactam, amoxicillin plus clavulanic acid) and broad spectrum cephalosporins (ceftriaxone). It has also been documented that vancomycin can contribute to the development of carbapenems resistance (100). Therefore, the present study suggests that antibiotic exposure might play a major role in the colonization of CPE, in agreement with other studies. (12, 50, 99)

Table 6 - Antibiotic exposure of *de novo* colonized patients

Antibiotic name	Patients No.	Patients %
Piperacillin + Tazobactam	19	26%
Amoxicillin + Clavulanic acid	14	19%
Ceftriaxone	7	10%
Vancomycin	5	7%
Ciprofloxacin	3	4%
Levofloxacin	3	4%
Trimethoprim/Sulfamethoxazole	3	4%
No antibiotics	3	4%
Azithromycin	2	3%
Clindamicin	2	3%
Flucloxacillin	2	3%
Metronidazole	2	3%
Amoxicillin	1	1%
Cefepime	1	1%
Cefixime	1	1%
Ceftaroline	1	1%
Clarithromycin	1	1%
Fosfomycin	1	1%
Gentamicin	1	1%
Tigecycline	1	1%
Total	73	100%

4.4. ANTIBIOTIC RESISTANCE PHENOTYPES AND CARBAPENEMASES

Considering the overall antimicrobial resistance rates of **infection isolates** (table 7), all isolates were resistant to third generation cephalosporins (100%), amoxicillin/clavulanic acid (100%) and piperacillin/tazobactam (100%). This result is expected and confirms that all isolates were multidrug resistant, particularly due to the extensive use of β -lactams and third generation cephalosporins in clinical practice.

Most isolates were non-susceptible to carbapenems, with ertapenem showing the highest resistance rate (92%), followed by imipenem (57%) and then meropenem (48%), which is in agreement with previous studies (30, 101).

The *E. coli* strain and almost all of the *K. pneumoniae* isolates were resistant to ciprofloxacin (91%). Gentamicin and fosfomycin showed low resistance rates (33% and 22%, respectively) and both are currently used as therapeutic options to treat CRE infections.

Table 7- Antibiotic resistance rates for infection isolates.

Antimicrobial category	Antimicrobial agent	<i>K. pneumoniae</i> (n=32)			<i>E. coli</i> (n=1)			<i>E. cloacae</i> complex (n=1)		
		T	I%	R%	T	I%	R%	T	I%	R%
Aminoglycosides	Amikacin	10	10%	0%	1	100%	0%	-	-	-
	Gentamicin	30	0%	33%	1	0%	100%	-	-	-
Antipseudomonal penicillins + β-lactamase inhibitors	Piperacillin / tazobactan	31	0%	100%	1	0%	100%	1	0%	100%
Carbapenems	Ertapenem	26	8%	92%	1	0%	0%	1	0%	100%
	Imipenem	21	33%	57%	1	0%	0%	1	0%	100%
	Meropenem	29	34%	48%	1	0%	0%	1	100%	0%
3rd generation cephalosporins	Ceftazidime	14	0%	100%	1	0%	100%	1	0%	100%
	Cefuroxime	31	0%	100%	1	0%	100%	1	0%	100%
4th generation cephalosporins	Cefepime	31	84%	16%	1	0%	100%	1	0%	100%
Fluoroquinolones	Ciprofloxacin	22	0%	91%	1	0%	100%	1	0%	0%
Folate pathway inhibitors	Trimethoprim / Sulfamethoxazole	24	0%	33%	-	-	-	-	-	-
Glycylcyclines	Tigecycline	16	6%	0%	1	0%	0%	1	0%	0%
Penicillins + β-lactamase inhibitors	Amoxicillin / clavulanic acid	32	0%	100%	1	0%	100%	1	0%	100%
Phosphonic acids	Fosfomycin	18	0%	22%	-	-	-	-	-	-

Only *K. pneumoniae* isolates were tested for trimethoprim/sulfamethoxazole and most of them were susceptible (33% R) to this antibiotic. Therefore, this result supports an increase in the use of trimethoprim/sulfamethoxazole as an alternative treatment of KPC-*K. pneumoniae* infections, alone or combined with colistin, in agreement with other studies (102, 103) .

Although cefepime had a lower resistance rate (16%), it was the antimicrobial with the highest intermediate score (84%) and none susceptible phenotype. All isolates tested were non-resistant to amikacin and tigecycline, which remain reliable therapeutic options.

The strain of *E. coli* produced an OXA-48 enzyme and showed no resistance to carbapenems, which can be explained by the weak hydrolysing activity of this

carbapenemase (13, 28). Some carbapenemase-producing isolates are categorized as susceptible with the current carbapenem breakpoints. (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_8.1_Breakpoint_Tables.pdf). Besides carbapenems, the only antimicrobial effective against this isolate was tigecycline.

The isolate of *E. cloacae* complex showed resistance to all antimicrobials tested, with the exception of ciprofloxacin and tigecycline.

Because of the worldwide emergence of colistin resistance and the recent identification of *mcr-1* gene in KPC-3-producing isolates in Portugal (61), colistin resistance genes were sought by PCR in representative isolates from different species, origins (infection, colonization) and lineages. None of the isolates tested revealed the presence of *mcr* genes (*mcr-1*, -2, -3, -4, -5); Moreover, the isolates described by Mendes *et al.* belonged to ST45 that exhibited the capsular type K24, which was not detected in the present study.

In agreement with the previous reports (35), KPC was the most prevalent carbapenemase (n=111/119; 93%), being identified among all bacterial species (table 8). However, OXA-48 (1%) was identified in one *E. coli* from a clinical isolate and VIM (1%) was found in one *E. cloacae* complex from a faecal sample. Clinical OXA-48-producing *Enterobacteriaceae* isolates were firstly described in Portugal in 2014 in *E. coli* and *E. cloacae* strains from the same patient (104). The presence of OXA-48 – producing-*E. coli* in this study may represent a risk of spread of these carbapenemases either in the hospital or subsequently in the community setting, and their possible persistence over time. The risk of dissemination and persistence in the community is even higher in this case, due to the fact that this isolate was presumptively associated with the problematic and endemic clone ST131. Also, the patient from which this *E. coli* clone was isolated had a positive screening 20 days ago, meaning that previous colonization by this clone was possible. Despite VIM carbapenemase harboured by *E. coli* and *K. pneumoniae* being prevalent in some endemic regions (like Greece), only sporadic isolates producing this carbapenemase had been reported in Portugal, namely from *K. pneumoniae* (105) and *K. oxytoca* (35, 106). The emergence of VIM-producing-*E. cloacae* has more recently been described in several countries (107, 108), what may explain the occurrence of the VIM isolate in this study.

Few isolates (n=6, 5%) belonging to *K. pneumoniae*, *E. coli* and *E. cloacae* complex produced carbapenemase (Blue-Carba positive) other than KPC, OXA-48, VIM, NDM, IMP or GES (multiplex and simplex PCR were negative for these carbapenemase families). These results suggest the possibility of a new or uncommon carbapenemase among these isolates that will be clarified in the future. The differences in the antibiotic susceptibility

profiles to β -lactams observed for some isolates suggest the possible circulation of different carbapenemase types (data not shown).

Table 8 - Distribution of carbapenemase enzymes among *Enterobacteriaceae* species.

Carbapenemase		Species	Source (no.)	
KPC	111	<i>K. pneumoniae</i>	95	C (63), I (32)
		<i>E. coli</i>	8	C (8)
		<i>K. oxytoca</i>	4	C (4)
		<i>E. cloacae</i> complex	3	C (2), I (1)
		<i>C. freundii</i> complex	1	C (1)
VIM	1	<i>E. cloacae</i> complex	1	C (1)
OXA-48	1	<i>E. coli</i>	1	I (1)
unknown	6	<i>K. pneumoniae</i>	3	C (3)
		<i>E. cloacae</i> complex	1	C (1)
		<i>E. coli</i>	2	C (2)

4.5. MOLECULAR EPIDEMIOLOGY OF *K. PNEUMONIAE* ISOLATES

The relationship among *K. pneumoniae* isolates (n=88) was tested by FTIR and capsular types inferred by comparison of spectra with our in-house models and further confirmed by *wzi* sequencing. This strategy allowed to infer a high diversity of lineages exhibiting variable capsular types, many of them corresponding to types correctly predicted by FTIR and previously described in Portugal.

Most isolates (n=51; 52%) were grouped by FT-IR and exhibited capsular type *wzi*64 (K64) which has been exclusively associated in our country with ST147, and all of them were KPC-3 producers (table 9). In fact, KPC-3 producing ST147 isolates were involved in a major outbreak that occurred in this hospital in 2015, when CPE emerged (109). This clone was responsible for 62.5% (n=20/32) of CPE infections in the present study, confirming the prevalence and successful dissemination of ST147 in this hospital during the whole period analysed. Approximately half of these isolates were identified in surgical wards (most of them causing infection) or in patients under decolonization protocol. All isolates identified in outpatients caused infection but in many of them there is no information on previous colonization. This lineage (ST147-K64) is known to be the most commonly involved in the dissemination of SHV-12 and subsequently KPC-3 in other Portuguese hospitals and healthcare institutions, including in the north of Portugal (122). It is an epidemic clone associated with the dissemination of CTX-M-15 in Hungary (110) and

particularly important in the global spread of multidrug resistance, namely due to carbapenemases (KPC-, OXA-48, VIM- and NDM-) (111-114). It was initially reported in Portugal associated with SHV-12 (115) and more recently with KPC-3 (30).

The other major group of isolates carried *wzi*187 which corresponds to KL27 (n=19; 19%), and produced KPC-3. It was mainly identified in medical wards especially in February 2018 (only one isolate causing infection in November 2017) and it was much more predominant among faecal samples (22%, n=15) than infection isolates (12,5%,n=4). Interestingly, most patients had record of negative results for previous colonization. The K locus 187 was associated in other studies with ST392 (116, 117). This sequence-type has been reported to have contributed to the dissemination of *bla*_{OXA-48} in Spain (101) and contributed to a nosocomial outbreak in China (*bla*_{KPC-2}) (118) and in Mexico (*bla*_{NDM-1})(119). Isolates with K-type KL27 and presumptive ST392 were not reported in the outbreak that occurred in this hospital a few years ago (109). This could indicate that a new clone of *K. pneumoniae* was introduced in the hospital environment and that it was capable to become the second most prevalent in the study, including among clinical isolates. To this date, this is the first study to report the emergence of KPC-3-producing *K. pneumoniae* associated with capsular-type KL27 in clinical isolates from Portugal, and adequate monitoring is needed.

Isolates presumptively related and exhibiting a variety of other capsular types were detected sporadically in both colonized and infected patients: *wzi*9-K9 (n=5), *wzi*173-KL102/KL149/KL155 (n=4), *wzi*93-KL112 (n=3), *wzi*53-K53 (n=2) and *wzi*50-K15K17K50K51K52 (n=2). Thus, many of these have been identified already before February 2018 causing infection, and subsequently in different patients as colonizers which suggests a diversity among the population of CPE currently causing infection. In colonized patients it was also found sporadic clones associated with *wzi*83-K23 (n=3), *wzi*19-K19 (n=2) and *wzi*102-K31 (n=1). One clinical isolate with *wzi*75-KL105 was also identified.

Similarly to what has been reported in previous studies of CPE in Portugal, the K-types often associated with the worldwide disseminated clone ST258 (31, 120) were not found.

Table 9 - Capsular type of *K. pneumoniae* strains, obtained by sequencing.

wzi allele (sequencing)	Predicted wzi (in house model)	No. of isolates			K-type	Carbapenemase detected	Date of isolation
		C	I	Total			
wzi64	wzi64	31	20	51	K14.K64. KL64	KPC (51)	04/11/17 - 27/02/18
wzi187	groups B/C	15	4	19	KL27	KPC (19)	29/11/17 - 27/02/18
wzi9	group A	4	1	5	K9.KL9	KPC (5)	09/01/18 - 13/02/18
wzi173	group D	3	1	4	KL102KL149KL155	KPC (4)	12/12/17 - 06/02/18
wzi93	wzi93	1	2	3	KL112	KPC (2), Other (1)	04/12/17 - 09/02/18
wzi83	wzi83	3	0	3	K23	KPC (3)	02/02/18 - 19/02/18
wzi53	group E	1	1	2	-	KPC (2)	31/01/18 - 15/02/18
wzi50	group F	1	1	2	-	KPC (2)	03/12/17 - 21/02/18
wzi102	wzi 94	1	0	1	K31	KPC (1)	02/02/18
wzi19	wzi19	2	0	2	K19	KPC (1), Other (1)	08/02/18 - 17/02/18
wzi75	outlier	0	1	1	-	KPC (1)	05/12/2017
unknown	outlier	4	1	5	-	KPC (4), Other (1)	18/12/17 - 15/02/18

Besides K64-(ST147) isolates, other capsular types that had been previously reported in Portugal associated with the dissemination of widespread clones such as ST15 (K19, KL112) or ST11 (KL105; wzi75) were also detected confirming that they still play a role on the MDR *K. pneumoniae* population that is circulating in Portuguese hospitals (16, 30, 35, 98). The K19 or KL112-ST15 lineages were detected earlier among KPC-producers and *K. pneumoniae* carrying OmpK36-modified porins (16, 30).

Only one isolate collected from a urine sample was identified as wzi75, which is associated with ST11. This clone is closely related to ST248 and was previously reported in KPC-producing *K. pneumoniae* among Portuguese patients (36, 121).

Other sporadic K-types (wzi93, wzi50, and wzi173) were found during the most part of the period (figure 28). On the other hand, K-types such as K9, K23, K31 or K53 (wzi53) were identified sporadically in more recent specimens. They were not detected in previous collections of carbapenemase-producing *K. pneumoniae* isolates in our country suggesting a more recent emergence. According to public databases (e.g. <http://bigsd.b.pasteur.fr/klebsiella/klebsiella.html>; KAPTIVE), these capsular types were associated with a variety of clones identified by multilocus sequence typing that seem not to be frequently detected.

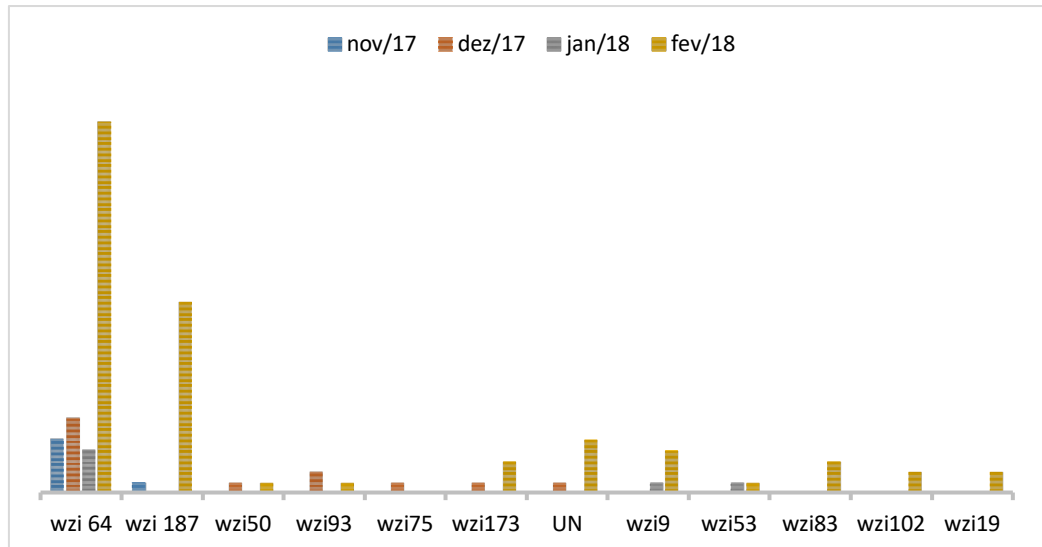


Figure 28 - Distribution of *K. pneumoniae* K-types during the time period of the study.

Regarding the two wards with the greatest incidence of CPE (Internal Medicine and Women Surgery), some differences concerning clonal diversity should be noted. First, the proportion between infection and colonization was high in Women Surgery (58% of infection samples), whereas the opposite was observed in Internal Medicine (67% of colonization samples). Most isolates recovered from Women Surgery belonged to K-type 64, either clinical or rectal ones. This can be observed not only in Women Surgery but in all surgical units (figure 29A). Since nearly all isolates belonged to the same clone, they probably have the same source, which suggests a transmission between patients or surface's contamination. On the other hand, most isolates (clinical and rectal) collected from Internal Medicine were associated with K-type KL27, followed by K64 (figure 29B). The higher number of screening isolates indicates that colonization has a major impact in this ward. Also, the variety of clonal types seems to point to a greater diversity of patients.

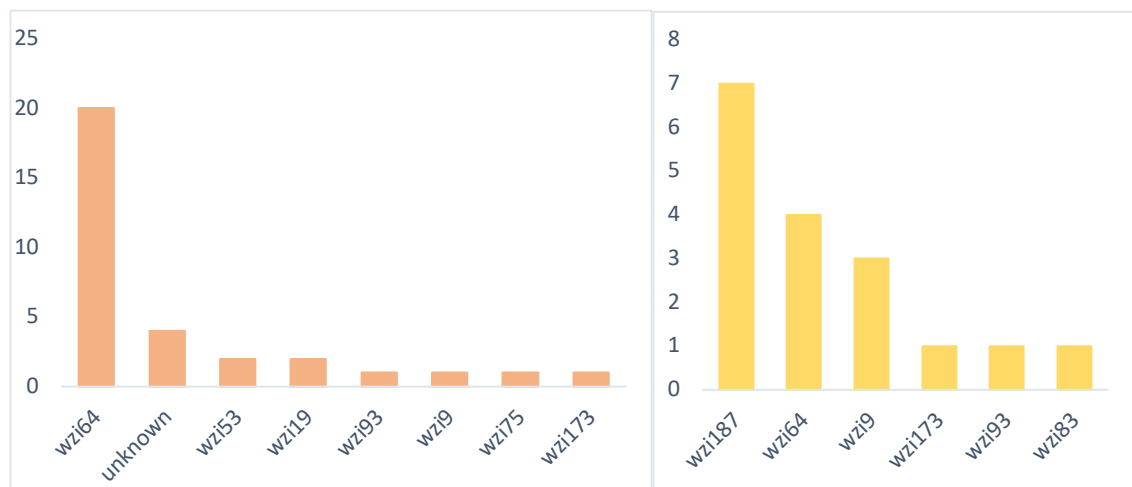


Figure 29 – (A) Clonal diversity of isolates from surgical wards; (B) clonal diversity in isolates from Internal Medicine

4.6. CHARACTERIZATION OF NON-*K. pneumoniae* ISOLATES

Regarding CPE isolates from species other than *K. pneumoniae* (table 10), *E. coli* was the microorganism most commonly found (n=11), followed by *E. cloacae* complex (n=6), *K. oxytoca* (n=4) and *C. freundii* complex (n=1). Only one isolate of *E. coli* (producing OXA-48) and other of *E. cloacae* complex (producing KPC) were found among infected patients, both collected from pus samples and in surgical wards. Moreover, these clinical isolates were collected more recently (January and February 2018).

Table 10 - Epidemiological characterization of CPE non- *K. pneumoniae* isolates.

Species (no.)	Source (no.)	Carbapenemase (no.)	Date of isolation	Gender (no.)	Age (mean)
<i>E. coli</i> (11)	Decolonization (6) Medical (5) Surgical (1)	KPC (8) OXA-48 (1) Others (2)	25/01/18 - 26/02/18	F (3) M (8)	42 - 87 (mean 64)
<i>E. cloacae</i> complex (6)	Surgical (5) Medical (1)	KPC (3) VIM (1) Others (2)	04/02/18 - 28/02/18	F (3) M (3)	38-86 (mean 69)
<i>K. oxytoca</i> (4)	Surgical (2) Medical (1) Decolonization (1)	KPC (4)	02/02/18- 22/02/18	F (3) M (1)	76 - 82 (mean 79)
<i>C. freundii</i> complex (1)	Surgical (1)	KPC (1)	12/02/2018	M (1)	20

Carbapenem-resistant *E. coli* belonged to distinct phylo-groups (table 11), being B2 the most prevalent one (n=5; 46%), followed by group E (n=2; 18%). All the other phylogenetic groups were represented (9% A, 9% B1, 9% C, 9% F) except for group D.

The strains identified as phylo-group B2 belonged mainly to the worldwide disseminated clone ST131 (n=4/5), which represented 36,3% (n=4/11) of all *E. coli* isolates. The ST131 isolates were most often found to harbour the *bla*_{KPC} gene (n=9; 81,8%). The *bla*_{OXA-48} is also present (n=1; 9%), being the only isolate with this carbapenemase type in the study. Potentially rare or new carbapenemases were also identified in *E. coli* isolates.

E. coli ST131 is a high-risk clone that is probably responsible for the global dissemination of *bla*_{KPC}-producing *E. coli* (122). The presence of this sequence-type in a healthcare facility in Portugal poses a major threat to public health because it has the potential to cause widespread resistance to carbapenems, including in the community setting.

Table 11 - *E. coli* phylo-groups.

No.	Date of collection	Sex	Age	Source	C / I	Philo-group	ST	Carbapenemase
1	20/02/2018	F	66	Rectal swab	C	A	nd	KPC-3
2	08/02/2018	M	69	Rectal swab	C	B1	nd	KPC-3
3	25/01/2018	M	42	Pus from abscess	I	B2	ST131	OXA-48
4	06/02/2018	M	50	Rectal swab	C	B2	ST131	KPC-3
5	15/02/2018	M	78	Rectal swab	C	B2	ST131	Other
6	17/02/2018	F	87	Rectal swab	C	B2	ST131	Other
7	21/02/2018	M	70	Rectal swab	C	B2	nd	KPC-3
8	08/02/2018	F	62	Rectal swab	C	C	nd	KPC-3
9	15/02/2018	M	55	Rectal swab	C	E	nd	KPC-3
10	19/02/2018	M	74	Rectal swab	C	E	nd	KPC-3
11	26/02/2018	M	53	Rectal swab	C	F	nd	KPC-3

*Other: negative for KPC, VIM, NDM, OXA-48 and IMP.

4.7. RELATIONSHIP BETWEEN SCREENINGS AND INFECTION ISOLATES

Considering infected patients with screenings history (figure 30), the days between colonization (first positive screening) and infection ranged from 0 to 421 days, with a mean of 44 days. Four of the 29 patients (14%) didn't have any positive screening before the isolate from infection. The others 25 patients (86%) were known CPE carriers before the collection of the clinical isolate.

From this group, five patients died, one of them as a result of an infection caused by a KPC-producing-*K. pneumoniae*, that was isolated from a tracheal aspirate. This patient (nº 10) was hospitalized during 16 days and was initially treated with amoxicillin plus clavulanic acid and piperacillin plus tazobactam. A CPE screening was performed 4 days after the admission and turned out negative. Three days later a KPC-producing *K. pneumoniae* was isolated and linezolid was administered. The patient passed away after 8 days of linezolid therapy. The isolate was susceptible only to amikacin, trimethoprim/sulfamethoxazol, imipenem and meropenem.

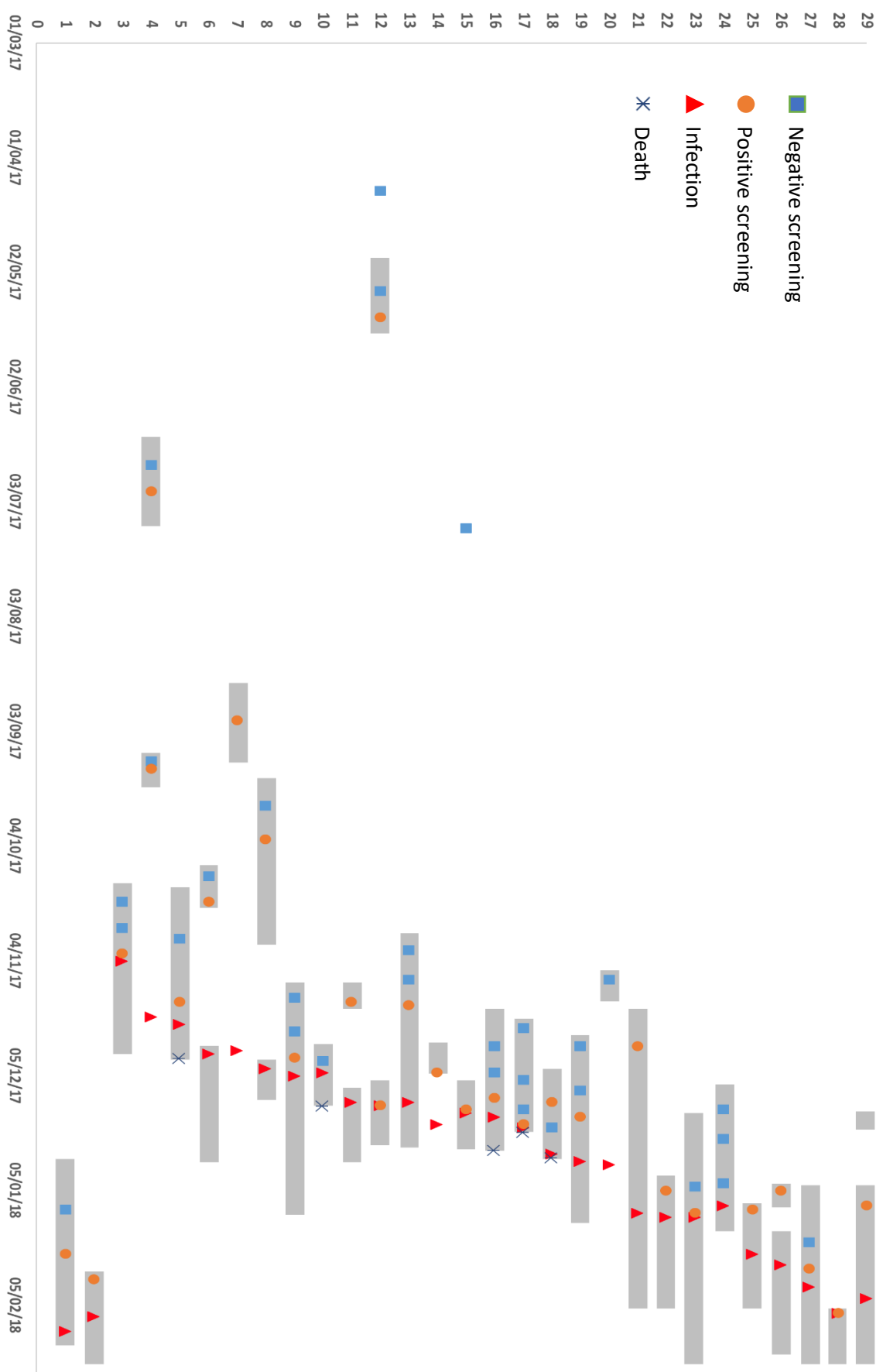


Figure 30 - Timeline of infected patients with screening history.

CHAPTER 5 - CONCLUSIONS

The emergence of CPE is a significant threat to hospitalized patients worldwide, mainly due to the scarcity of therapeutic options for treating infections with MDR bacteria. The dissemination of KPC-3 among *K. pneumoniae* isolates became recently evident in Portugal, leading to an implementation of reinforced infection control measures and active surveillance in the hospitals (30, 35). Despite the low number of CPE in invasive infections, Portugal was one of the participating countries in EuSCAPE that showed greater proportions of KPC-positive *K. pneumoniae* (123). Although the transmission of CPE is increasing at an alarming pace, little is known about the effectiveness of these infection control measures or about the relationship between clinical and screening isolates, highlighting the relevance of this study.

Overall, the results of this dissertation conducted to the following general conclusion:

This study alerts for the current dissemination of a high diversity of CPE infecting or colonizing patients hospitalized in this hospital, and underlines the importance of a close monitoring of the infection control to contain the spread of emerging clones and carbapenemases.

Particular conclusions are summarized as follows:

- The current CPE population in this hospital comprises a variety of species (*K. pneumoniae*, *E. coli*, *E. cloacae* complex, *K. oxytoca* and *C. freundii* complex), *K. pneumoniae* (diverse capsular types) and carbapenemases (although KPC-3 is still predominant). The majority of patients seem to have acquired the CPE infecting or colonizing isolate in the nosocomial setting, which reinforces the relevance of implementation of infection control measures in the hospital environment to prevent MDR-bacteria dissemination.
- Internal medicine and women surgery were the hospital wards with higher percentage of CPE isolates, underlining the need of re-evaluating and monitoring infection control practices, particularly among professionals from these areas.
- Long hospitalization periods and antibiotic exposure were identified as possible factors that seems to have contributed to CPE colonization.
- Decolonization by FMT was effective in 88% of the patients but they all became recolonized. Further studies and more information about this measure may be useful to conclude more about its long-term effectiveness.

- Carbapenemase-producing *K. pneumoniae* presented a diversity of capsular types that are presumptively associated with a diversity of clones. Overall, the capsular types K64 (n=51) and KL27 (n=19) were the most prevalent types in both colonization and infection samples.
- After the initial outbreak in 2015 in this hospital, *K. pneumoniae* K64 (ST147) has successfully disseminated throughout the hospital and was responsible for the majority of infections, some with fatal outcomes. Notwithstanding other unknown factors, an inefficient infection control seems to have driven its expansion.
- The high number of recent isolates identified as *K. pneumoniae* KL27 may indicate that a new and successful clone is emerging and disseminating in the hospital environment.
- The acquisition of carbapenemases (KPC and OXA-48) by the successful *E. coli* lineage ST131 may increase the threat of carbapenemase-producing-*E. coli*, due to its importance in the community setting.

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