INSTITUTO POLITÉCNICO DE LISBOA

ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA

GLOBAL DIVERSITY, PHYLOGENETIC ANALYSIS AND MOLECULAR DETECTION OF *bla*$_{KPC}$ AND *bla*$_{OXA}$ GENES.

Catarina Dos Santos Marques Nunes

Supervisor (s):

Prof. Doutor João Ruben Lucas Mota Perdigão. Faculdade de Farmácia da Universidade de Lisboa, Universidade de Lisboa.

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Mestrado em Tecnologias Moleculares em Saúde

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Global diversity, phylogenetic analysis and molecular detection of *bla*$_{KPC}$ and *bla*$_{OXA}$ genes

Catarina Nunes

2023

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Abstract

Bacterial infections are a worldwide concern. Strains responsible for these infections, such as carbapenem-resistant-*Enterobacterales*, have acquired a wide variety of carbapenemases becoming resistant to a large range of carbapenems, especially *Klebsiella pneumoniae* strains. In these species, the prominent genes coding for carbapenemases appears to be bla_{KPC} and bla_{OXA}. As a result, this project focused on global diversity and phylogenetic analyses, as well as on the molecular detection of these two genes.

For phylogenetic diversity analyses, we obtained 88 bla_{KPC} and 428 bla_{OXA} reference sequences from the NCBI. These sequences were then used in a programmatic tblastn against the entire nucleotide database at NCBI for taxonomic dispersion analyses, yielding 1232 bla_{KPC} and 2204 bla_{OXA} alleles. Three KPC groups and five OXA groups could thus be established. Furthermore, we discovered three Tn*4401* isoforms (Tn*4401a*, Tn*4401b*, and Tn*4401d*) linked to 450 bla_{KPC}, as well as a $bla_{\mathrm{KPC\text{-}31}}$ gene found in a Tn*4401d*. A $bla_{\mathrm{OXA\text{-}51}}$ gene was discovered in *Salmonella*, and five $bla_{\mathrm{OXA\text{-}58}}$ genes in *Proteus*. Furthermore, using herein-designed oligonucleotides, bla_{KPC} or bla_{OXA} genes were successfully amplified from 52 *Enterobacterales* isolates. Also, no correlation was found between the carriage of $bla_{\mathrm{KPC\text{-}181}}$ and altered susceptibility to ceftazidime-avibactam.

Overall, we were able to establish previously unreported KPC groups, identify a $bla_{\mathrm{KPC\text{-}31}}$ gene within a Tn*4401d,* and the presence of a $bla_{\mathrm{OXA\text{-}51}}$ gene in *Salmonella*. Additionally, a specific multiplex PCR detection method was designed and implemented for bla_{KPC} and $bla_{\mathrm{OXA\text{-}48\text{-}like}}$ while also establishing no correlation between the emergence of $bla_{\mathrm{OXA\text{-}181}}$ and decreased susceptibility to ceftazidime-avibactam.

Keywords

KPC; OXA; CRE; *Klebsiella pneumoniae*; Tn*4401*

Resumo

As infeções bacterianas são um problema global. As estirpes responsáveis por essas infeções, como as *Enterobacterales* resistentes a carbapenemos, têm vindo a adquirir vários genes que codificam para carbapenemases, particularmente as estirpes de *Klebsiella pneumoniae*. Nesta espécie, os genes mais relevantes parecem ser os *bla*$_{KPC}$ e *bla*$_{OXA}$. Consequentemente, este projeto focou-se na diversidade global e análise filogenética, bem como na deteção molecular desses dois genes.

A partir das análises de diversidade filogenética, obtivemos do NCBI 88 *bla*$_{KPC}$ e 428 *bla*$_{OXA}$ sequências de referência. Essas sequências foram usadas num tblastn programático contra todo o banco de dados nucleotídico do NCBI para a análise de dispersão taxonómica, tendo-se obtido 1232 e 2204 sequencias *bla*$_{KPC}$ e *bla*$_{OXA}$, respetivamente. Foi possível identificar três grupos de *bla*$_{KPC}$ e cinco grupos de *bla*$_{OXA}$, tendo sido identificadas três isoformas de Tn*4401* (Tn*4401a*, Tn*4401b*, and Tn*4401d*) associadas a 450 *bla*$_{KPC}$. Identificou-se ainda um gene *bla*$_{KPC-31}$ posicionado num Tn*4401d*. Um gene *bla*$_{OXA-51}$ foi identificado em *Salmonella* e cinco genes *bla*$_{OXA-58}$ em *Proteus*. Adicionalmente, utilizando primers desenhados neste estudo, ambos os genes foram amplificados com sucesso em 52 isolados de *Enterobacterales* tendo-se ainda verificado a ausência de correlação entre o gene *bla*$_{OXA-181}$ e menos suscetibilidade à ceftazidima-avibactam.

Concluindo, foi possível estabelecer grupos de *bla*$_{KPC}$ não relatados anteriormente, identificar um gene *bla*$_{KPC-31}$ num Tn*4401d* e um gene *bla*$_{OXA-51}$ em *Salmonella*. Suplementarmente, foi desenhado e implementado um PCR-multiplex para a deteção da presença de genes *bla*$_{KPC}$ e *bla*$_{OXA-48-like}$ estabelecendo também a ausência de relação entre o gene *bla*$_{OXA-181}$ e diminuição da suscetibilidade à ceftazidima-avibactam.

Palavras-chave

KPC; OXA; CRE; *Klebsiella pneumoniae*; Tn*4401*

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List of Abbreviations

KPC	*Klebsiella pneumoniae* carbapenemases
KPC-*Kp*	*Klebsiella pneumoniae*-producing carbapenemases
OXA	Oxacillinases
AMR	Antimicrobial resistance
MDR	Multidrug resistance
XDR	Extensively drug resistant
CDC	Centers for Disease Control and Prevention
PBPs	Penicillin-binding proteins
ESBLs	Extended-spectrum β-lactamases
MBLs	Metallo β-lactamases
IMP	Imipenemase-metallo-β-lactamases
VIM	Verona integron encoded metallo-β-lactamases (VIM)
NDM	New Delhi metallo-β-lactamases
CRE	Carbapenem-resistant *Enterobacterales*
NTE	Non-transposon elements
MLST	Multilocus sequence typing
ECDC	European Centre for Disease Prevention and Control
CZA	Ceftazidime–avibactam
HGT	Horizontal gene transfer
MRSA	Methicillin-resistant *Staphylococcus aureus*
OMP	Outer membrane protein
MGEs	Mobile genetic elements

1. Introduction

Throughout the course of time, bacterial pathogens and associated infections have emerged as a primary concern worldwide (1). Antiseptic procedures have been implemented to reduce mortality associated with these infections but the key role in reducing infectious disease-associated mortality has been mainly due to the introduction of antimicrobial agents (2).

Antimicrobial agents are compounds, derived from any source (microorganisms, plants, animals, synthetic or semi-synthetic) that at low concentrations can suppress and inhibit the growth of microorganisms. This class of agents can be designated as antibacterial (antibiotics and chemotherapeutics against bacteria), antiviral (against viruses), antifungal (against fungi), and antiparasitic (against parasites) (3). The first antimicrobial agent was salvarsan, developed in 1910, against syphilis and synthesized by Paul Ehrlich. In 1928, the first antibiotic, penicillin, was discovered by Alexander Fleming and came into clinical use in the 1940s. The discovery of penicillin changed the course of the battle of humans against infectious diseases, at least for a while. In fact, penicillin is an outstanding agent in terms of efficacy and safety and led to the era of antibacterial chemotherapy by saving the lives of many wounded soldiers during World War II (4).

Additionally, following those discoveries, a larger number of antimicrobial agents, mostly antibacterial, have been found over the years (**Figure 1.1**).

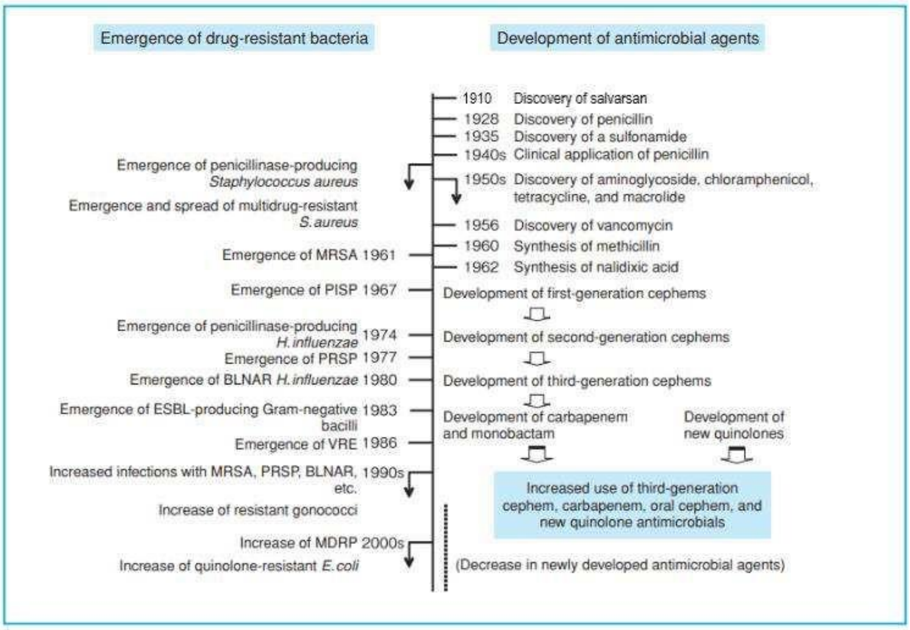

Figure 1.1- Trend of development of antimicrobial agents and emergence of drug-resistant bacteria over the years. **Adapted from:** (4).

1.1. Antimicrobial Consumption

Antimicrobial agents have become a valuable tool employed on a large scale and in many sectors, leading to a large consumption of these agents. In 1987 the antimicrobial consumption in animal feeds in the United States was 75% of the nation's annual consumption (5). Likewise, in 2014, the average antimicrobial consumption in Europe was higher in animals (152 mg/kg) than in humans (124 mg/kg) (6).

The total consumption of antibacterial agents for systemic use, in all countries of the European Union (EU) and European Economic Area (EEA) is listed in **Table 1.1**. The values are expressed as the number of defined daily doses (DDD) per 1000 inhabitants per day.

Table 1.1- Total consumption (community and hospital sector) of antibacterial for systemic use by country (expressed as DDD per 1 000 inhabitants per day). **Abbreviations:** EU/EEA- European Union/European Economic Area; - total care data (community and hospital sector) not reported. **Adapted from**: (7).

Country	*2012*	*2014*	*2016*	*2018*	*2020*
Austria					8.8
Belgium	25.6	24.0	24.2	22.3	16.6
Bulgaria	17.4	20.0	19.2	21.1	22.7
Croatia	20.0	19.4	18.7	18.8	15.7
Cyprus	25.1	22.2	28.4	28.0	28.9
Czechia					13.4
Denmark	17.4	17.1	17.0	15.6	14.3
Estonia	12.2	11.9	12.0	11.8	10.5
Finland	20.6	19.1	17.4	15.4	11.9
France	25.7	24.9	25.6	25.3	20.3
Germany					
Greece	29.9	31.0	33.1	34.1	28.1
Hungary	14.1	15.2	14.4	14.8	11.2
Iceland	19.7				
Ireland	21.0	21.0	22.0	22.4	18.6
Italy	24.6	24.5	24.0	21.4	18.4
Latvia	12.9	12.6	12.9	13.4	12.3
Lithuania	15.3	15.1	16.6	16.3	14.1
Luxembourg	25.0	23.2	22.9	22.1	16.1
Malta	20.8	22.4	20.9	20.2	16.6
Netherlands	10.9	10.3	10.1	9.7	8.5
Norway	17.9	16.9	16.2	15.3	13.9
Poland		21.2	22.0	24.4	18.5
Portugal	20.1	18.0	19.0	19.1	15.2
Romania	25.9	26.6	24.4	25.1	25.2
Slovakia	19.7	21.2	23.6	22.0	14.4
Slovenia	13.2	13.1	13.0	13.2	10.2
Spain	15.7	17.1	27.5	26.3	19.8
Sweden	15.3	14.0	13.2	12.4	10.4
EU/EEA	**21.2**	**21.2**	**20.9**	**20.3**	**16.4**
United Kingdom		20.8	19.7	18.8	

1.2. Antimicrobial Resistance (AMR)

The wide use of antimicrobial agents, over the years, has, on the other hand, imposed and/or strengthened an additional selective pressure that has led to the emergence of antimicrobial resistance (AMR). Microorganisms have revealed a remarkable ability to adapt, evolve, and survive by developing resistance to every therapeutic compound (8). According to WHO, AMR occurs when microorganisms change over time and become able to withstand the presence of drugs that once impacted them (9).

In order to improve the comparability of surveillance data for these organisms and to more accurately assess their epidemiological significance and public health impact, it has also become important to adopt standardized international terminology to define resistant organisms to a significant number of therapeutically active drugs. Therefore, different classes were created for different types of AMR: multidrug-resistance (MDR), extensively drug-resistance (XDR), and pandrug-resistance (PDR) (**Figure 1.2**). The MDR organisms are defined as resistant to three or more antimicrobial classes, as well as resistant to one key antimicrobial agent. Meanwhile, XDR organisms can be resistant to many or nearly all approved antimicrobial agents, if not all. On the other hand, PDR can have different definitions, such as resistant to almost all commercially available antimicrobials, resistant to all antimicrobials routinely tested, or/and resistant to all antibiotic classes available for empirical treatment (10).

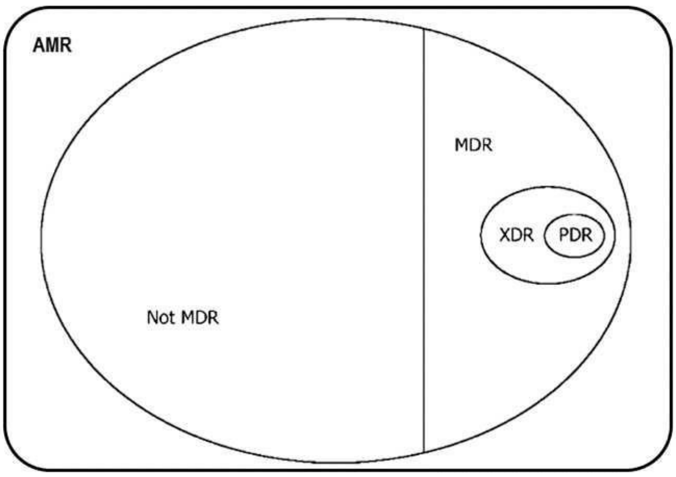

Figure 1.2- Diagram illustrating the relationship between MDR, XDR, and PDR. **Adapted from:** (10).

The use of these AMR definitions enables a global overview of the global AMR burden, which poses not only a nonnegligible economic toll but also a public health issue. In 2007, the economic burden of AMR in Europe was estimated to be at least 1.5 billion euros, with hospital costs accounting for 900 million (11), while estimates from 2014 predicted that Europe is expected to have 390.000 AMR-related casualties by 2050 **(Figure 1.3)** (12).

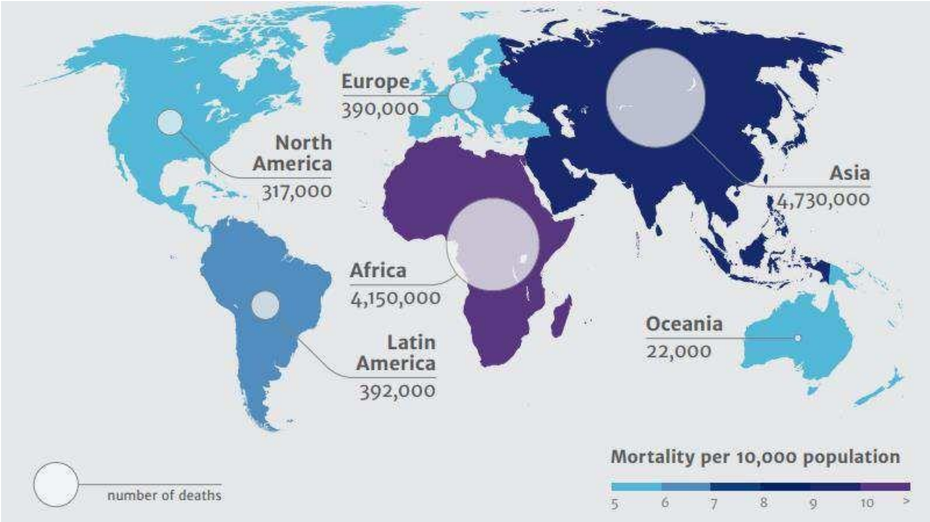

Figure 1.3- AMR attributable deaths every year by 2050. **Adapted from:** (12).

1.2.1. <u>Epidemiology of AMR and ESKAPE pathogens</u>

Antimicrobial resistance has spread throughout the world, with MDR bacteria posing the greatest threat. Associated with high levels of MDR and widespread worldwide are a group of pathogens known as the ESKAPE. ESKAPE stands for ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa*, and ***E****nterobacter spp*. (13,14).

Among these, and a major focus for this thesis, stands *Klebsiella pneumoniae*, a Gram-negative bacillus member of the *Enterobacterales* order, that is encapsulated, rod-shaped, and is a facultative anaerobic bacterium (15). Epidemiologically, this pathogen is the most prominent among the carbapenem-resistant *Enterobacterales* (CRE) (16). The polymyxins, tigecycline, third-generation cephalosporins or carbapenems class of antibiotics, or the more recent ceftazidime-avibactam (CZA) combination are the mainstay of treatment for serious infections caused by this bacteria (17,18).

According to a 2021 World Health Organization (WHO) report, more than ten pathogens are classified as pathogens of priority level, which includes all the ESKAPE pathogens (**Table 1.2**) (19).

Table 1.2- WHO priority pathogens list for 2021. **Abbreviations:** ESBL- extended-spectrum β-lactamases. **Adapted from:** (19).

	Pathogen priority level
Medium	*Streptococcus pneumoniae*, penicillin-non-susceptible
	Haemophilus influenzae, ampicillin-resistant
	Shigella spp., fluoroquinolone-resistant
High	*Neisseria gonorrhoeae,* cephalosporin-resistant, fluoroquinolone-resistant
	Salmonellae, fluoroquinolone-resistant
	Campylobacter spp., fluoroquinolone-resistant
	Helicobacter pylori, clarithromycin-resistant
	Staphylococcus aureus, methicillin-resistant, vancomycin-intermediate and resistant
	Enterococcus faecium, vancomycin-resistant
Critical	carbapenem-resistant *Enterobacterales* (CRE), ESBL-producing
	Pseudomonas aeruginosa, carbapenem-resistant
	Acinetobacter baumannii, carbapenem-resistant

The carbapenem-resistant *Enterobacterales* as well as the carbapenem-resistant pathogens *A. baumannii* and *P. aeruginosa*, are critical-level priority pathogens. Since *K. pneumoniae* is the most widespread CRE, the data rates addressed below only apply to carbapenem-resistant *K. pneumoniae* and the two other critical pathogens (carbapenem-resistant *P. aeruginosa* and carbapenem-resistant *A. baumannii*). Carbapenem resistance data on these three pathogens, from seven countries worldwide, are summarized in **Table 1.3**.

Table 1.3- Carbapenem resistance data from seven different countries worldwide (China, USA, UK, Spain, Portugal, South Africa, and India) concerning the three most critical pathogens (*A. baumannii* carbapenem-resistant, *P. aeruginosa* carbapenem-resistant, and *K. pneumoniae* carbapenem-resistant). **Gathered from:** China Antimicrobial Surveillance Network (CHINET); European Antimicrobial Resistance Surveillance Network (EARS-Net); Global Antimicrobial Resistance Surveillance System (GLASS); Becton Dickinson (BD); National Healthcare Safety Network, US (NHSN); Indian Council of Medical Research (ICMR); Fortis Healthcare Limited; South African Society for Clinical Microbiology (SASCM); National Health Laboratory Service (NHLS). **Adapted from:** (20).

Pathogens	***Country***	***Antibiotics***	***Resistance Percentage (invasive isolates)***	***Year***
Acinetobacter baumannii	China	Carbapenems	82	2017
	India	Carbapenems	71	2019
	Portugal	**Carbapenems**	**31**	**2019**
	South Africa	Carbapenems	80	2019
	Spain	Carbapenems	58	2019
	UK	Carbapenems	4	2019
	USA	Carbapenems	30	2016
Klebsiella pneumoniae	China	Carbapenems	36	2017
	India	Carbapenems	65	2014-2019
	Portugal	**Carbapenems**	**12**	**2019**
	South Africa	Carbapenems	18	2016-2019
	Spain	Carbapenems	7	2019

	UK	Carbapenems	2	2019
	USA	Carbapenems	3	2012-2016
Pseudomonas aeruginosa	China	Carbapenems	25	2017
	India	Carbapenems	30	2009-2017
	Portugal	**Carbapenems**	**19**	**2019**
	South Africa	Carbapenems	30	2014-2016
	Spain	Carbapenems	25	2019
	UK	Carbapenems	8	2019
	USA	Carbapenems	10	2011-2016

At the European level and according to the European Centre for Disease Prevention and Control (ECDC) data for 2021, Greece (73.7%) and Romania (54.5%) have the highest number of carbapenem-resistant *K. pneumoniae* isolates in Europe, while Portugal (11.6%) is ranked ninth (**Figure 1.4)** (21).

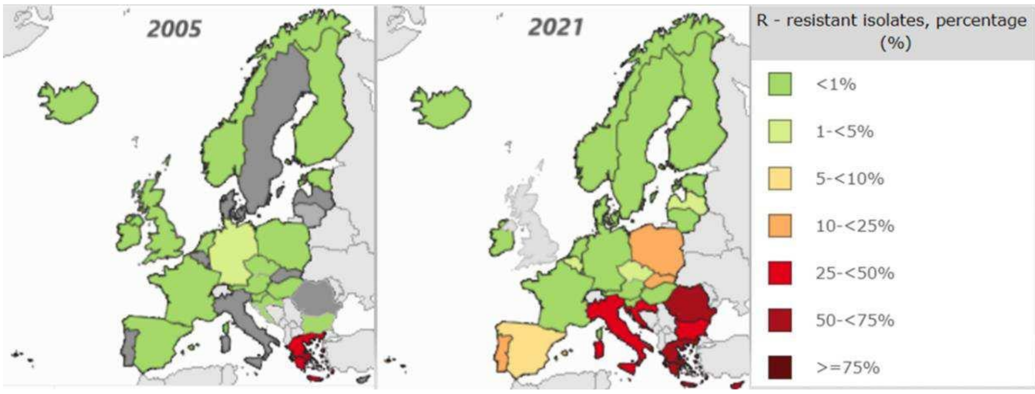

Figure 1.4- Representation of surveillance atlas of infectious diseases. Data rates of carbapenem-resistant *K. pneumoniae* isolates, in Europe. Countries in gray have not applicable (N/A) rates. **Data rates (%):** 2021: Austria- 1.0%; Belgium- 1.4%; Bulgaria- 46.3%; Croatia- 32.9%; Cyprus- 26.2%; Czechia- 1.0%; Denmark- 0.5%; Estonia- 0.9%; Finland- 0.0%; France- 0.8%; Germany- 0.8%; Greece- 73.7%; Hungary- 0.9%; Iceland- 0.0%; Ireland- 0.6%; Italy- 26.7%; Latvia- 1.6%; Lithuania- 1.0%; Luxembourg- 1.0%; Malta- 6.7%; Netherlands- 0.2%; Norway- 0.3%; Poland- 19.5%; Portugal- 11.6%; Romania- 54.5%; Slovakia- 11.7%; Slovenia- 0.9%; Spain- 5.9%; Sweden- 0.2%. 2005: Austria- 0.0%; Belgium- N/A; Bulgaria- 0.0%; Croatia- 0.0%; Cyprus- N/A; Czechia- 0.0%; Denmark- N/A; Estonia- 0.0%; Finland- 0.0%; France- 0.1%; Germany- 2.0%; Greece- 27.8%; Hungary- 0.3%; Iceland- 0.0%; Ireland- 0.0%; Italy- N/A; Latvia- N/A; Lithuania- N/A; Luxembourg- N/A; Malta- N/A; Netherlands- 0.0%; Norway- 0.6%; Poland- 0.0%; Portugal- N/A; Romania- N/A; Slovakia- N/A; Slovenia- 0.0%; Spain- 0.0%; Sweden- N/A; United Kingdom- 0.0%. **Adapted from:** (21).

1.3. Mechanisms of AMR and spectrum of activity

Given the global significance of ESKAPE pathogens and their epidemiology, these pathogens developed distinct resistance mechanisms that make them resistant to nearly all antibiotics, with most of them being MDR (14). These resistance mechanisms may be native to the microorganisms or acquired from other microorganisms. Native resistance can be intrinsic (always expressed in the species) or induced (the genes are naturally occurring in the bacteria but are only expressed after exposure to an antibiotic). On the other hand, acquired resistance develops as a result of chromosomal DNA mutations or the acquisition of genetic material through horizontal gene transfer (HGT). Plasmid-mediated transmission of resistance genes is the most common route of HGT (22). These plasmids can obtain, accumulate and shuttle a great variety of resistance genes through transposons, integrins, and insertion sequences (IS), enabling its horizontal transfer to other strains or species and thus easily and quickly disseminate antibiotic resistance activity in a population (23). Resistance mechanisms have evolved to the point where resistant bacteria can modify antibiotic binding sites/targets, reduce antibiotic uptake and accumulation (via porins and efflux pumps), and inactivate antibiotic molecules (**Table 1.4**)(24–26).

Table 1.4- Mechanisms of antibiotic resistance. **Abbreviations:** PBPs- penicillin-binding proteins, MDR-multidrug resistance. **Adapted from:** (27).

<table>
<tr><th colspan="2">Resistance Mechanism</th><th>Antibiotic commonly affected</th></tr>
<tr><td rowspan="6">Modification of antibiotic binding site/target</td><td>Mutation in PBPs proteins</td><td>Penicillins</td></tr>
<tr><td>Mutation in protein in 30S ribosomal subunit</td><td>Aminoglycosides (e.g. streptomycin)</td></tr>
<tr><td>Replace alanine with lactase in peptidoglycan</td><td>Vancomycin</td></tr>
<tr><td>Mutation in DNA-gyrase</td><td>Quinolones</td></tr>
<tr><td>Mutation in RNA polymerase</td><td>Rifampin</td></tr>
<tr><td>Mutation in catalase-peroxidase</td><td>Isoniazid</td></tr>
<tr><td rowspan="2">Reduce antibiotic uptake and accumulation</td><td>Mutation in porin proteins</td><td>Penicillins, aminoglycosides</td></tr>
<tr><td>MDR pump</td><td>Tetracyclines, sulfonamides, quinolones</td></tr>
<tr><td>Antibiotic molecule inactivation/alteration</td><td>Cleavage by β-lactam</td><td>β-lactam antibiotic</td></tr>
</table>

1.3.1. Modification of antibiotic's binding site

Modification of antibiotic binding sites is one of the most common mechanisms of antibiotic resistance in bacteria. This mechanism can occur by reducing the affinity or preventing the antibiotic molecule from binding. Numerous components in bacterial cells may be antimicrobial agent targets, as well as numerous targets that bacteria may modify to enable antibiotic resistance. Therefore, the binding site and its modification differ depending on the antibiotic used. The most frequently prescribed antibiotics worldwide are the β-lactams (28).

The β-lactam antibiotic family includes four different antibiotic classes, penicillins, cephalosporins, monobactams, and carbapenems (**Figure 1.5**). The carbapenems are the most potent β-lactam antibiotic among these classes (29). The modification of β-lactam binding sites is accomplished through mutations in the genes encoding for penicillin-binding proteins (PBPs). These proteins stand in the cytoplasmic membrane **(Figure 1.6**) and can be class A PBPs, a bifunctional protein with glycosyltransferases and transpeptidases activity (involved in mediating peptidoglycan cross-linking in the cell wall), or a monofunctional class B PBPs with only transpeptidase activity (30).

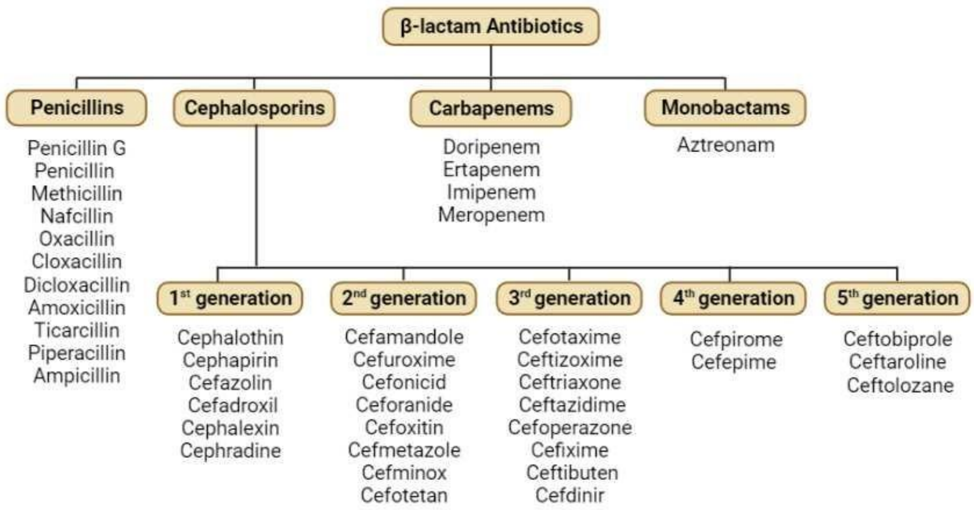

Figure 1.5- Schematic overview of all β-lactam antibiotic classes clinically available. **Gathered from:** (31,32). **Created from:** (33).

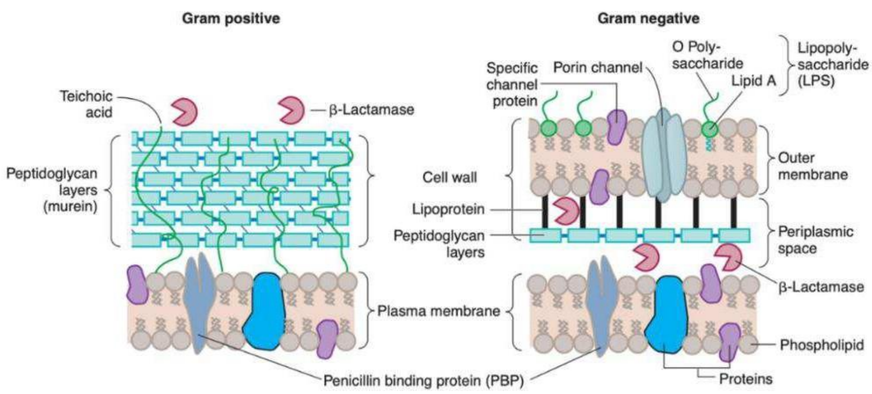

Figure 1.6- Structure and composition of gram-positive and gram-negative cell walls. Representation of PBP position in cell walls. **Adapted from:** (34).

The PBPs proteins typically are responsible for the assembly of the peptidoglycan portion of the bacterial cell wall (15). Peptidoglycans are essential for cell shape, growth, division, and viability. For that same reason, the interaction between β-lactam molecules and PBPs proteins prevents the capacity of PBPs to catalyze peptidoglycan assembly, leading to an improper formation of the peptidoglycan layer inducing cell lysis and death by changing its osmotic pressure (35,36).

Nonetheless, AMR bacteria can avoid this β-lactam mechanism of action through mutations in their binding sites (PBPs), and thus develop a resistance mechanism. This resistance mechanism is exemplified by the methicillin-resistant *S. aureus* (MRSA) strain, which is resistant to methicillin and virtually all β-lactam antibiotics but some fifth-generation cephalosporins such as ceftaroline. MRSA harbours the *mecA* gene, which encodes for PBP2a, a modified PBP with a low affinity for β-lactams (37), enabling the survival of *S. aureus* in the presence of high concentrations of β-lactam antibiotics. Furthermore, both *E. faecalis* and *E. faecium* express PBP5, a low-affinity chromosomally encoded ortholog of PBP2a in MRSA that confers low to moderate β-lactam resistance (38). Resistance to imipenem owing to hyperproduction of an altered PBP5 with reduced affinity for this agent has also been seen in *E. faecium* (39). Another common alternative PBP is the penicillin-binding protein 2x (PBP2x), which was isolated from clinical β-lactam-resistant *Streptococcus pneumoniae* isolates. This mutated protein has a reduced affinity for β-lactam antibiotics and their transpeptidase domain carries numerous substitutions compared with homologous sequences from β-lactam-sensitive *Streptococcus* (40).

1.3.2. Reduce antibiotic uptake and accumulation

One strategy used by bacteria to develop antibiotic resistance is to reduce the number of molecule antibiotics that can pass through the bacterial cell membrane. This can occur due to a reduced number of protein channels (porins), and/or the presence of efflux pumps, which reduces the amount of drug accumulated within the cells (15).

1.3.2.1. Porins

Porins are protein channels capable of allowing the passage of many hydrophilic substances, including antibiotics. Mutations in porins can modify and reduce porin expression, as well as lead to the loss of these outer membrane protein (OMP) channels. As many different drugs frequently share the same porin, this type of resistance usually results in multiple antibiotic resistance that may cross antibiotic class lines (28). Some examples are the resistance to imipenem in *Pseudomonas aeruginosa* that arises from expression reduction of porin protein OprD, leading to decreased imipenem influx into the cell (41). Furthermore, insertional inactivation of OprD porin also reduces carbapenem susceptibility in *P. aeruginosa* isolates (42). Another pathogen, *A. baumannii*, developed carbapenem resistance due to the loss of a 29-kDa OMP and/or expression of CarO, Omp33-36, and OprD porins (43). Additionally, the porin OmpA has been associated with *A. baumannii* resistance to cephalothin and cephaloridine, due to its low-permeability channel (44).

1.3.2.2. Efflux pumps

The expression of efflux pumps actively extrude antibiotic out of the cell, greatly contributing this way to AMR. Genes encoding efflux pumps can be located on the chromosome or within mobile genetic elements (MGEs) (38). To date, six major families of efflux pumps have been characterized: resistance-nodulation-division (RND), major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE), small multidrug resistance (SMR), ATP-binding cassette (ABC), and proteobacterial antimicrobial compound efflux (PACE) families (25,45).

The overexpression of RND multidrug efflux pumps (MexAB–OprM, MexCD-OprJ, MexXYOprM) results in resistance against β-lactam, extended-spectrum cephalosporins, tetracyclines, fluoroquinolones, and aminoglycosides, in *P. aeruginosa* strains (46,47). Moreover, RND efflux pumps in *K. pneumoniae* and *E. aerogenes* strains can cause imipenem, quinolone, and chloramphenicol resistance, and some RND homologues cause relevant tigecycline resistance (48,49). The *A. baumannii* isolates can also demonstrate an MDR phenotype through the presence and overexpression of efflux pump ABC. This pump is associated with resistance to fluoroquinolones, *β*-lactams, tetracyclines, macrolides, chloramphenicol, and aminoglycosides (15).

1.3.3. Antibiotic molecule inactivation/alteration

Many bacteria produce enzymes that irreversibly inactivate/modify antibiotics. The most common one is through β-lactamases, which are highly prevalent and extremely diverse. This mechanism has extremely high efficiency and constitutes the major antibiotic-resistant mechanism in the bacterial world. Therefore, to overcome this resistance, β-lactam antibiotics are often given with β-lactamase inhibitors (50).

The β-lactamase enzymes responsible for β-lactam-resistance, act by hydrolyzing the β-lactam antibiotics binding themselves and opening the β-lactam ring, consequently disrupting its antibiotic activity **(Figure 1.7**) (15).

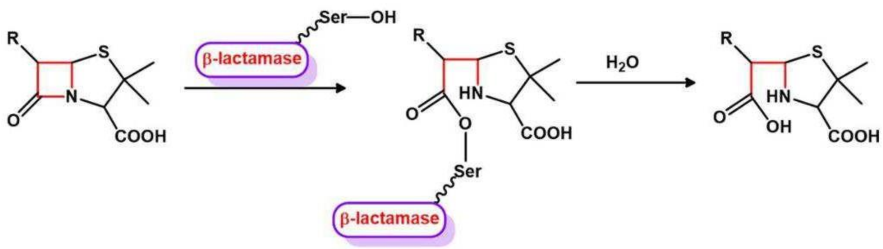

Figure 1.7- A schematic representation of serine-based class A and class C β-lactamases hydrolyzing the β-lactam antibiotic. **Adapted from:** (51).

Currently, several bacteria have developed numerous β-lactamases, including the ESBL, AmpC cephalosporinases, and carbapenemases (29). The β-lactamases can be categorized using two main classification systems: the Ambler system, based on their sequence similarity, and the Bush system, based on their functionality (15,52,53).

1.3.3.1. Ambler classification

The Ambler classification groups β-lactamases into four different molecular classes (A, B, C, and D). Classes A, C, and D involve a serine ester hydrolysis mechanism, whereas class B, has a zinc ion participating in catalysis (**Figure 1.8**) (52,53).

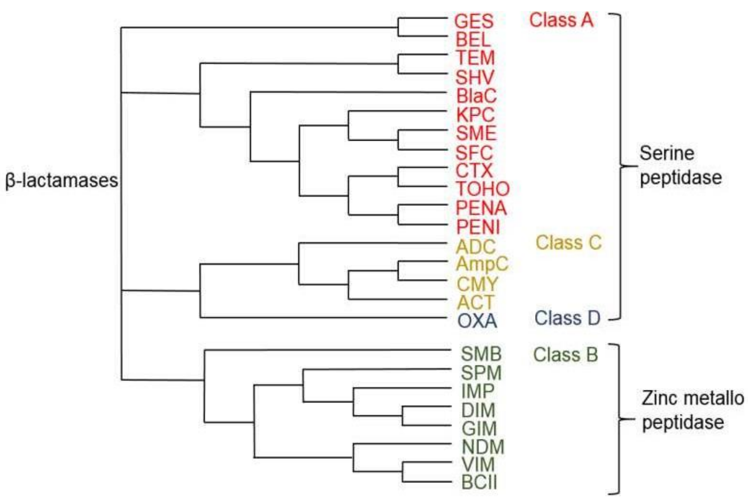

Figure 1.8- Phylogenetic relationships among all β-lactamases by Ambler classification. **Adapted from:** (54).

Class A was the first β-lactamase crystal structure to be determined from *Staphylococcus aureus* in 1940 (**Figure 1.9**). This class of enzymes consists of a diverse array of narrow-spectrum β-lactamases, ESBLs enzymes, and carbapenemases. Narrow-spectrum β-lactamases hydrolyze penicillins (except temocillin), ESBLs hydrolyze narrow and ESBL-antibiotics, while the carbapenemases hydrolyze carbapenems (55). Moreover, this class hydrolyzed also the cephalosporins and monobactam antibiotics (56). To date, over 2.000 class A β-lactamases have been identified. The majority of them belong to functional subtypes of gram-negative enzymes such as TEM, CTX-M, KPC, or CARBA (57). Some of these class A β-lactamases are chromosome-encoded (NmcA, SME, IMI-1, SFC-1) and others plasmid-encoded (*Klebsiella pneumoniae* carbapenemases (KPC), IMI-2, GES, GES derivatives) (58). All β-lactamases include in this class share a serine peptidase at their active site which confers the hydrolytic property to these enzymes.

Class B includes metallo-β-lactamases (MBLs), which require a zinc ion as a cofactor. Bacteria that produce this class of enzymes show resistance to all β-lactam antibiotics, except aztreonam. MBLs enzymes are plasmid-encoded, thus they are easily transmitted to other microorganisms. The most common MBLs enzymes are imipenemase-metallo-β-lactamases (IMP), verona integron encoded metallo-β-lactamases (VIM), and the New Delhi metallo-β-lactamases (NDM) (15). The IMP type of enzymes has mainly been found in *P. aeruginosa*, *K. pneumoniae*, *A. baumannii,* and *E. cloacae*, whereas the VIM type has been detected mostly in *P. aeruginosa* and *A. baumannii*. On the other hand, NDM has been discovered in *E. cloacae* and *K. pneumoniae* (53,59).

The **class C** enzymes comprise penicillinases and cephalosporinases. Among the cephalosporinases, the chromosomally encoded AmpC is clinically important and is typically found in *P. aeruginosa* and *Enterobacterales* family, such as *Enterobacter spp*. and *K. pneumoniae*. The AmpC β-lactamases inactivate aztreonam, all penicillin, and most cephalosporins antibiotics. Furthermore, the AmpC enzyme is resistant to most β-lactamases inhibitors except avibactam (60). Avibactam is a covalent, slowly reversible inhibitor with a structure that does not contain a β-lactam core but maintains the capacity to covalently acylate its β-lactamase targets (61).

Class D enzymes involve a variety of β-lactamases called oxacillinases (OXA). This class of enzymes was initially characterized by their ability to efficiently hydrolyze isoxazolyl-type β-lactams like oxacillin. The hydrolytic activity of OXAs is mainly against penicillins and 1st generation cephalosporins (62). Nevertheless, there are OXA enzymes capable of hydrolyzing carbapenems, known as carbapenem-hydrolyzing class D β-lactamases (CHDLs), which possess a weak activity against ESBL-cephalosporins and do not hydrolyze monobactams (63). Furthermore, OXA-type carbapenemases are commonly found in *A. baumannii*, which have low catalytic efficiency, and together with porin deletion and other antibiotic resistance mechanisms, can cause a severe high resistance to carbapenems (62,64). Although most OXA enzymes are resistant to β-lactamase inhibitors, OXA-18 and OXA-45 are well inhibited by clavulanic acid (65,66).

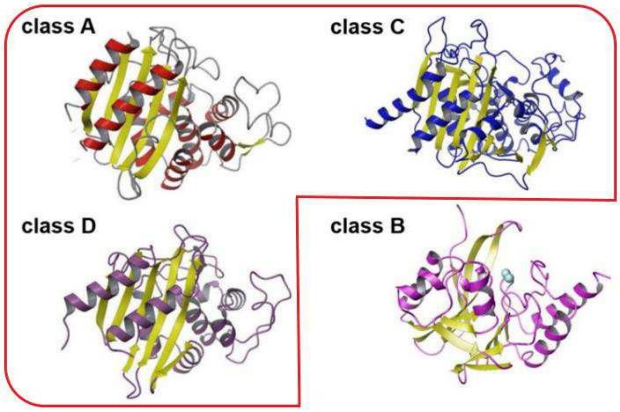

Figure 1.9- Schematic crystal structure of β-lactamase classes. The red line highlights the class A, C and D belonging to serine peptidase group. Class A (TEM-1; PDB ID: 1JTG), class C (AmpC; PDB ID: 2PU4), class D (OXA-23; PDB ID: 4JF6) and class B (VIM-2; PDB ID: 4BZ3). **Legend:** PDB- Protein Data Bank **Adapted from:** (54,67).

1.3.3.2. <u>Bush classification</u>

The Bush classification uses three groups to classify β-lactamases: group 1 (cephalosphorinases), group 2 (serine β-lactamases), and group 3 (MBLs), each of which is further subdivided (**Figure 1.10**; **Table 1.5**).

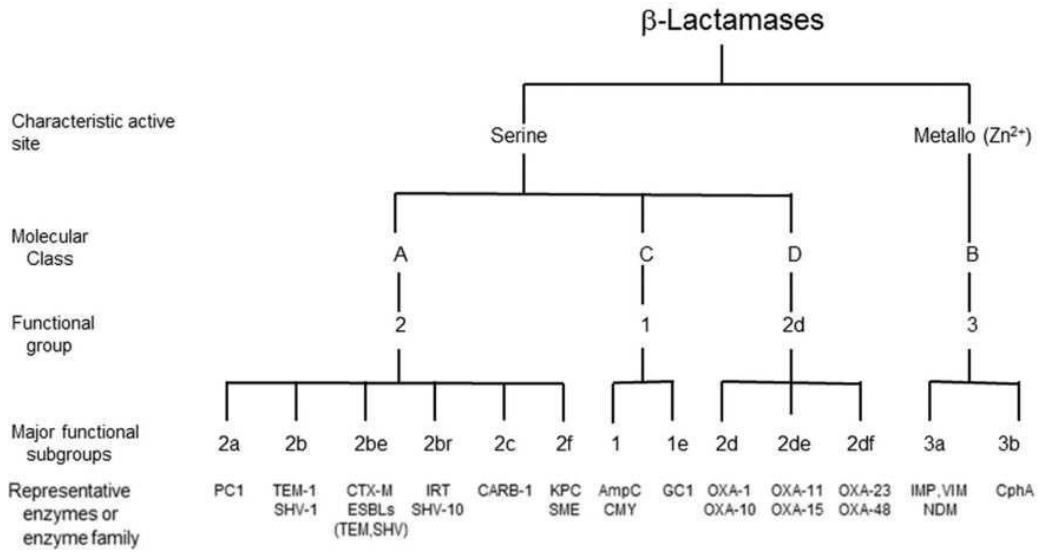

Figure 1.10- Schematic molecular and functional features of the major β-lactamases groups. Molecular classes are based on Ambler system, and functional groups/subgroups on Bush system. **Adapted from:** (52,68).

Table 1.5- Classification of β-lactamases by Bush and Ambler systems and description of defining characteristics for each group/class and their representative enzymes (53).

Bush classification	Ambler classification	Distinctive substrates	Defining characteristics	Representative enzymes
1	C	Cephalosporins	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e			Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	GC1, CMY-37
2a	A	Penicillins	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b		Penicillins, early cephalosporins	Similar hydrolysis between benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be		ES-cephalosporins, monobactams	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br		Penicillins	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber		ES-cephalosporins, monobactams	Increased hydrolysis of oxyimino-β-lactams, and resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c		Carbenicillin	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce		Carbenicillin, cefepime	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	D	Cloxacillin	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de		ES-cephalosporins	Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams	OXA-11, OXA-15
2df		Carbapenems	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	A	ES-cephalosporins	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f		Carbapenems	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	B	Carbapenems	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, IND-1, CcrA, L1, CAU-1, GOB-1, FEZ-1
3b		Carbapenems	Preferential hydrolysis of carbapenems	CphA, Sfh-1

In **Group 1 (class C)** the enzymes are cephalosporinases encoded on the chromosomes of many *Enterobacterales* and a few other organisms (69). Some Group 1 β-lactamases are extended-spectrum AmpC (ESAC), while others are plasmid-mediated ESBLs, CMY, ACT, DHA, FOX, MIR, and GC1 (53,60). This group is poorly inhibited by clavulanic acid or sulbactam (β-lactamase inhibitors) (70).

Regarding **Group 2 (class A and D)**, these enzymes are serine β-lactamases (penicillinases, cephalosporinases, and ESBL) that generally are inhibited by clavulanic acid and tazobactam (53,69). The majority of this group's β-lactamases are chromosomal, though some are plasmid-encoded. The TEM, SHV, ESBLs, KPC, SME, CARB, PC1, CTX-M, IRT, IMI, NMC, GES, and OXA enzymes are among them (52,53,71). Benzylpenicillin and many other penicillin derivatives are preferentially hydrolyzed by this group. Cephalosporins, carbapenems, and monobactams, on the other hand, are poorly hydrolyzed by these enzymes (53). These β-lactamases are prevalent in gram-positive pathogens such as *Staphylococci* and occasionally *Enterococci* (72) and in gram-negative pathogens such as *Enterobacterales* (71), particularly *K. pneumoniae*, which has already acquired a massive variety of β-lactamase enzymes, with KPC and OXA being the most common (73).

Group 3 (class B) are MBLs that hydrolyze penicillins, cephalosporins, and carbapenems and are poorly inhibited by almost all β-lactam antibiotics (69). MBLs enzymes originally were identified as chromosomal in gram-positive or occasional gram-negative bacilli, such as *Bacteroides fragilis* (74) or *Stenotrophomonas maltophilia* (75). This group differs structurally from the other groups by their requirement for a zinc ion at the active site. Functionally, they were once distinguished primarily by their ability to hydrolyze carbapenems, but some serine β-lactamases (group 2) have also acquired this ability. In contrast to group 2 β-lactamases, the MBLs have poor hydrolytic capability for monobactams and are not inhibited by clavulanic acid or tazobactam (53). Instead, they are inhibited by metal ion chelators including EDTA, dipicolinic acid, or phenanthroline (76).

1.4. Resistance to carbapenems

The emergence and spread of carbapenem-resistant bacterial pathogens have become a worldwide public health issue in recent decades (77). The mechanism of resistance through β-lactamases expression poses a threat and is responsible for acquired resistance in species usually exhibiting full susceptibility to carbapenems, such as all species belonging to the *Enterobacterales order*, *Acinetobacter baumannii,* and *Pseudomonas aeruginosa* (78). Consequently, even if several intensive infection control practices are used, outbreaks of carbapenemase-mediated MDR strains are only reduced and cannot be completely eradicated (38).

Carbapenemase enzymes have versatile hydrolytic capacities, with the ability to hydrolyze penicillins, cephalosporins, monobactams, and carbapenems. There are three main groups of carbapenemases, KPC, MBLs, and OXA (79). These enzymes have been identified among the Ambler classes, corresponding to class A (KPC), class B (MBLs), and class D (OXA).

For class C enzymes, there is a controversy on whether carbapenemases exist in this group or not. Some articles claim that doesn't exist (53,80,81), while more recent articles report the existence of three enzymes that could potentially be classified as carbapenemases (CMY, ACT, ADC) due to an overproduction of AmpC that leads to a significant carbapenemases activity (77,82).

1.4.1. <u>Class A carbapenemases</u>

The class A carbapenemases group includes *Serratia marcescens* enzyme (SME), imipenem hydrolysing beta-lactamase (IMI), not metallo enzyme carbapenemase (NMC), Guiana extended-spectrum (GES) and KPC families. KPC carbapenemases are the most common and their coding genes are frequently found on plasmids in *K. pneumoniae* (83). Plasmids and transposons, designated as MGEs, are the main vectors enabling and facilitating the spread of carbapenemases (84). The bla_{KPC} genes are frequently inserted into a Tn*4401* transposon element (10-kb) and less frequently into a non-transposon element (NTE), as the non-Tn*4401* variants I-II (79,85).

This genetic element (Tn*4401*) contains a transposase gene (*tnpA*), a resolvase gene (*tnpR*), and two insertion sequences, designated IS*Kpn6* and IS*Kpn7* **(Figure 1.11**). Eight isoforms of Tn*4401* transposon have been described thus far (Tn*4401*a [99bp deletion], Tn*4401*b [no deletion], Tn*4401*c [215bp deletion], Tn*4401*d [68bp deletion and -5.3kb deletion], Tn*4401*e [255bp deletion and lacking tnpA and tnpR], Tn*4401*f [215bp deletion, truncated tnpA and lacking tnpR, IS*Kpn*7 and Tn*4401* IRL], Tn*4401*g [215bp deletion in the noncoding region and lacking tnpA, tnpR and IS*Kpn*7] and Tn*4401*h [188bp deletion]). The Tn*4401*a and Tn*4401*b isoforms are the most prevalent (86). Additionally, the noncoding region between IS*Kpn*7 and bla_{KPC} was proposed to contain three putative promoter regions (P1, P2, and P3). P1 (absent in isoforms c and d) and P2 (present in all isoforms, with a 35bp deletion located inside the right inverted repeat of ISKpn7) were demonstrated to affect expression, while P3 was shown not to be a real promoter (87).

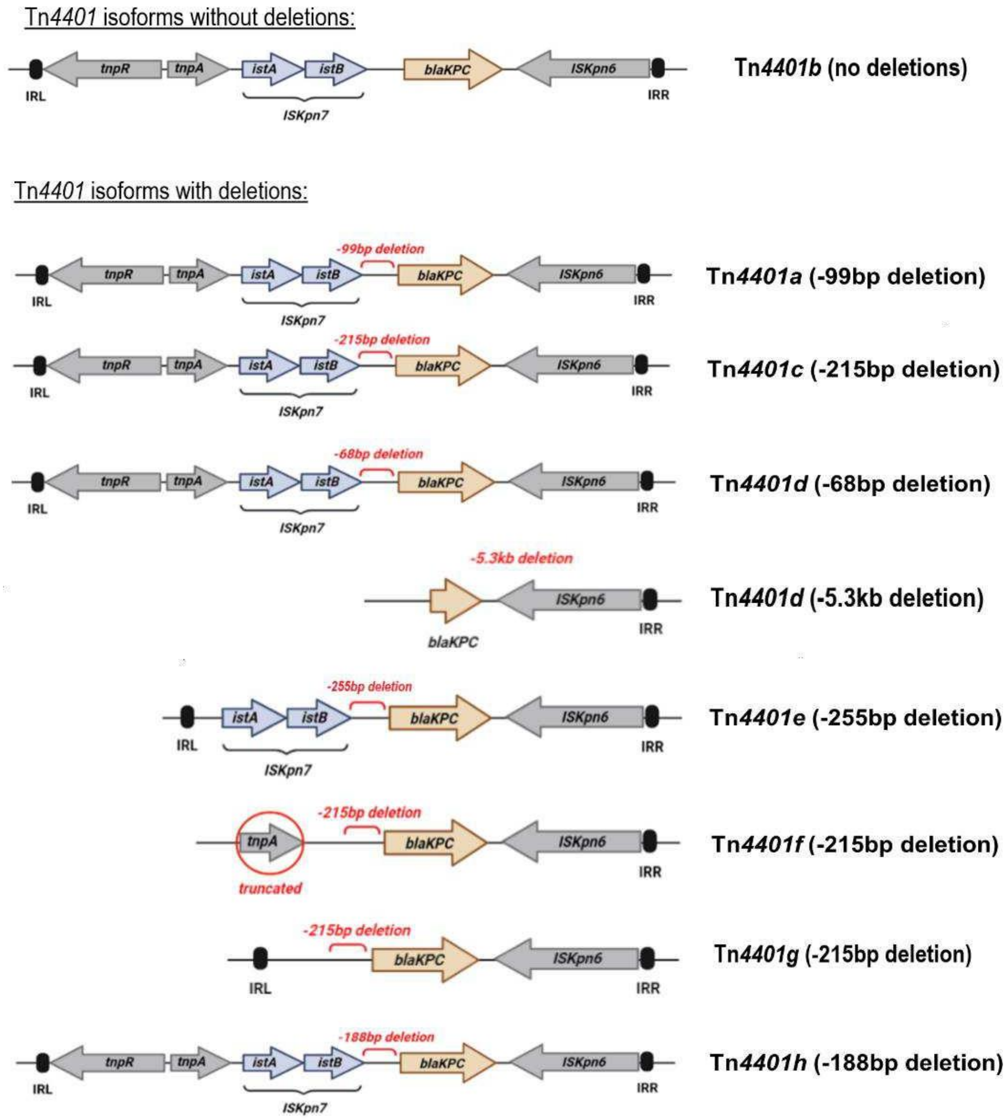

Figure 1.11- Schematic representation of all Tn*4401* isoforms known until date (a, b, c, d, e, f, g and h). Protein coding sequences are represented by the arrows and labeled with gene names or products. **Information from:** (86). **Created from:** (33).

The bacterial genera that have been identified as harbouring these transposon isoforms in the literature, are listed in **Table 1.6**.

Table1.6- Bacterial genera reported for each Tn*4401* transposon isoform.

Tn*4401* isoform	**Bacteria reported with Tn*4401***	**References**
Tn*4401a*	*Klebsiella pneumoniae*	(86,88)
	Escherichia coli	
	Citrobacter spp.	
	Morganella morganii	
	Serratia marcescens	
Tn*4401b*	*Klebsiella pneumoniae*	(86,89–91)
	Escherichia coli	
	Pseudomonas aeruginosa	
	Enterobacter cloacae	
	Klebsiella variicola	
	Klebsiella oxytoca	
	Citrobacter	
Tn*4401c*	*Klebsiella pneumoniae*	(92)
	Enterobacter cloacae	
	Escherichia coli	
Tn*4401d*	*Klebsiella pneumoniae*	(93,94)
	Raoultella	
Tn*4401e*	*Klebsiella pneumoniae*	(95)
Tn*4401f*	*Klebsiella pneumoniae*	(96)
Tn*4401g*	*Escherichia coli*	(97)
	Citrobacter freundii	
	Klebsiella oxytoca	
Tn*4401h*	*Enterobacter cloacae*	(86,98)
	Escherichia coli	
	Acinetobacter baumannii	

1.4.1.1. KPC Epidemiology

KPC enzymes display a wide geographical variation, with several clones disseminating within the same area but differing by their multilocus sequence typing (MLST-type), plasmid size, and β-lactamase enzyme content (99). By 2013, a previous study had already described 12 alleles of the *bla*$_{KPC}$ gene, which encodes the KPC enzyme (81). Nonetheless, 88 *bla*$_{KPC}$ alleles are described in the NCBI reference gene catalogue in 2022 (100). This enzyme, in addition to *K. pneumoniae*, has already been identified in *Enterobacter spp*., *E. coli*, *Klebsiella oxytoca*, and *C. freundii* (101), along with *P. aeruginosa*, and *A. baumannii* (102,103).

The worldwide dissemination of KPC-*Kp* has been associated mainly with the transposon Tn*4401*. Nevertheless, the NTE-KPC-ST258 is also a high-risk clone responsible for 80% of KPC-*Kp* outbreaks in the United States and about 90% of infections in Israel. The first NTE-KPC was described in China in 2007, and additional NTEs with different structures were later described in Argentina, Colombia, Chile, and

Brazil (104). In Portugal, the first isolate of KPC-*Kp* was discovered at a Lisbon hospital in 2009. Since then, single cases and sporadic outbreaks have been reported and the country faced an increasing trend of carbapenem resistance among *K. pneumoniae* from 0.8% in 2008 until reaching 11.6% in 2021 (21,80). Many studies on the level of KPC molecular epidemiology report KPC-2 and KPC-3 as the most common enzymes produced by the *Enterobacterales* order globally (105,106).

1.4.2. Class D carbapenemases

Class D carbapenemases are OXA-type β-lactamases frequently found in *A. baumannii* strains (83). The CHDLs enzymes belonging to this class, and have been reported almost exclusively from *A. baumannii*, worldwide (107).

The first OXAs (OXA-1, OXA-2, and OXA-3) were plasmid-encoded and were identified in Gram-negative bacteria, which were mainly penicillinases (77), while the first ESBL-OXA variant (OXA-11), exhibited a transferrable resistance profile with enhanced ceftazidime hydrolysis (108). Over the years new ESBL-OXA enzymes appeared, OXA-13 and OXA-14 (109), as well as a few variants of OXA-2 (OXA-4, OXA-5, OXA-6, and OXA-15) (110,111). Additionally, some OXA-10 mutants have also been reported (OXA-16 and OXA-17) (112), as well as OXA-19, OXA-28, and OXA-45 in *P. aeruginosa* isolates (66,113,114).

Nowadays, according to NCBI metadata, OXA enzymes are comprised of 5 groups, OXA-23-like, OXA-24/40-like, OXA-48-like, OXA-51-like, and OXA-58-like (115). Some studies reveal that OXA-48-like carbapenemases are the predominant OXA among *Enterobacterales* isolates (116). While OXA-23-like and OXA-51-like are the most found in *A. baumannii* isolates (117–121).

The $\textit{bla}_{\text{OXA-like}}$ genes weakly hydrolyze carbapenems, despite their capacity to confer high resistance when overexpressed in association with MGE (122). The transposon Tn*2006* is the most frequently observed MGE associated with $\textit{bla}_{\text{OXA}}$ genes worldwide. An example is the $\textit{bla}_{\text{OXA-23}}$ genes, which are usually located on plasmids and are flanked by two copies of the insertion sequences (IS*Aba1*) in opposite directions on the transmissible transposons Tn*2006* and Tn*2008*. They can also exist in association with the transposon Tn*2007*, which lacks the second copy of IS*Aba1*, and is associated with only one copy of IS*Aba4* (**Figure 1.12)** (77).

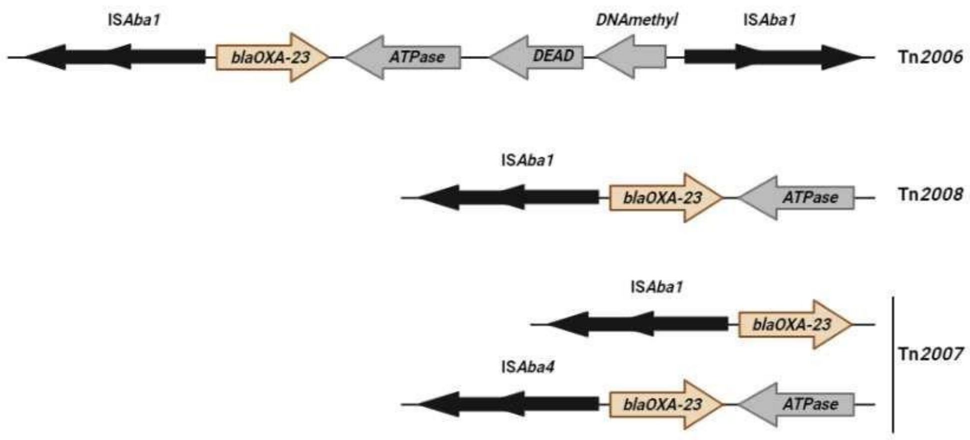

Figure 1.12- Schematic representation of Tn*2006*, Tn*2007* and Tn*2008* transposons harboring a *bla*$_{OXA-23}$ gene. Protein coding sequences are represented by the arrows and labeled with gene names or products. **Information from:** (123). **Created from:** (33).

1.4.2.1. OXA Epidemiology

Healthcare-associated carbapenem-resistant *A. baumannii* (CRAB) infections are a serious threat associated with global epidemic clones and especially OXA carbapenemase enzymes.

The majority of OXA carbapenemases have been discovered in *Acinetobacter spp.* (OXA-23-like, OXA-24/40-like, OXA-51-like, and OXA-58-like), being the OXA-51-like family the highly prevalent among this genus.

Although most OXA enzymes have been discovered in *Acinetobacter spp.*, there are some of them also found in other bacterial genera, especially in *Enterobacterales* (108). The carbapenemase-producing *Enterobacterales* (CPE) are rare in the United States but quite prevalent in Europe, including several European countries such as France, Germany, The Netherlands, Italy, Belgium, the UK, Ireland, Slovenia, Switzerland, Romania, Spain and Portugal (124,125). A study from 2020 revealed that the first two cases of OXA-48-producing *Enterobacterales* were found in *E. cloacae* and *E. coli* isolates, in Portugal, as well as the emergence of OXA-48 in *K. pneumoniae* isolates, mostly reported with the sequence type (ST) ST*11* (126). Moreover, a more recent study, carried out in Lisbon revealed the rise of an OXA-48-like enzyme (OXA-181), which has been increasing during the last few years (80).

1.5. Objectives

Nosocomial bacterial infections are a major focus of concern worldwide. In the last decade, strains responsible for these infections, such as carbapenem-resistant *Enterobacterales*, have become resistant to a large range of carbapenems, and have been extensively reported in *Klebsiella pneumoniae* strains (127). *K. pneumoniae* has acquired over time a wide variety of carbapenemase enzymes responsible for resistance against carbapenems. The prominent carbapenemase enzymes in these species appear to be KPC and OXA.

In Portugal, the carbapenem resistance in *K. pnueomoniae* strains increased from 0.7% in 2008 to 11.6% in 2021, being the ninth European country with the highest rates of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) (21). The common KPC enzymes reported in *K. pneumoniae* comprise the KPC-2 and KPC-3 (105,106,128), while the OXA enzymes identified in this genus are mostly the OXA-48-like family, recently reported a rise in an OXA-48-like enzyme (OXA-181) in clinical strains in Portugal (80,129).

Therefore, the objective of this master thesis was to investigate the overall genetic diversity, evolutionary trajectory, and taxonomical dissemination of KPC and OXA enzymes. Concomitantly, this thesis also attempted to test the capacity of a set of primers to amplify $\textit{bla}_{\text{KPC-3}}$ and $\textit{bla}_{\text{OXA-48-like}}$ genes ($\textit{bla}_{\text{OXA-48}}$ and $\textit{bla}_{\text{OXA-181}}$), as well as to test the implementation of a multiplex PCR detection in a single PCR reaction.

2. Materials and Methods

2.1. Diversity and taxonomical distribution of KPC and OXA coding genes

The phylogenetic analysis included in this study was performed across all KPC and OXA coding genes (*bla*$_{KPC}$ and *bla*$_{OXA}$ respectively) available in NCBI Reference Gene Catalog for antimicrobial resistance genes (100). In total, 88 alleles of the *bla*$_{KPC}$ gene and 428 alleles of the *bla*$_{OXA}$ gene were obtained. These sequences were also filtered by their carbapenemase activity: 73 out of 88 *bla*$_{KPC}$ were annotated as having carbapenemase activity whereas 240 out of the 428 *bla*$_{OXA}$ alleles were classified as carbapenemases (**Table 3.1**). The *bla*$_{OXA}$ allele sequences were stratified across five different groups according to the available NCBI metadata: OXA-23-like, OXA-24/40-like, OXA-48-like, OXA-51-like, and OXA-58-like.

For phylogenetic diversity analysis of both genes (*bla*$_{OXA}$ and *bla*$_{KPC}$), the nucleotide sequences obtained from NCBI were downloaded through the Batch Entrez NCBI and then aligned in MAFFT software version 7 (130). Following that, we performed a phylogenetic reconstruction using the IQ-TREE software version 2.1.3 (131). The phylogenetic tree annotations were performed using the Interactive Tree Of Life (ITOL) version 6.5.2 (132,133), while MEGA version 11 software was used for phylogenetic tree visualization and extraction of specific subtrees (134).

Furthermore, taxonomical distribution analysis was performed using a programmatic tblastn for each allele against the entire nucleotide database at NCBI, yielding a total of 1232 *bla*$_{KPC}$ and 2204 *bla*$_{OXA}$ alleles. Hits displaying identity (aminoacidic level) and coverage below 100% were excluded and taxonomical annotation was carried out using the annotate_blast_hits.py script (available at (135)). Subsequent phylogenetic analyses on all hits were performed by extracting the full nucleotide coding sequences using the hit accession and blast coordinates through custom R scripts and the Entrez Programming Utilities (E-utilities) interface to the NCBI database system. Extracted sequences were aligned using MAFFT and phylogenetic reconstruction was carried out using IQTree2. Tree annotation and visualization were carried out as previously described.

2.1.1. Genetic context of *bla*$_{KPC}$ genes

The genetic context associated with each *bla*$_{KPC}$ hit was characterized by extracting the *bla*$_{KPC}$ gene from each accession record identified along with up to an additional 2000 bp flanking regions. Initial characterization was carried out using ABRicate (135,136) along with an inhouse database comprehending the flanking regions of Tn*4401* isoforms a, b, and d. Concomitantly, retained sequences were aligned using MAFFT software version 7 (130). These analyses were redone when necessary, using different groups of sequences to acquire a database file with information about the transposon isoforms, as well as the coverage and identity percentage. All the sequences with less than 100% of coverage and 99% of identity were discarded. The resulting alignments were inspected using the Nucleotide BLAST-NCBI tool (137) and the NCBI nucleotide graphics to confirm the Tn*4401* isoforms obtained. Concurrently, the Bioedit software (138) was used to confirm the deletions associated with each Tn*4401* isoform.

Upon identification and comparison of the signature sequences associated with each Tn*4401* isoform, a second characterization using ABRicate-bio.tools was carried out using an updated inhouse database.

2.2. Molecular detection of *bla*$_{KPC-3}$ and *bla*$_{OXA-48-like}$ genes in *Klebsiella pneumoniae* clinical isolates

The detection of *bla*$_{KPC}$ and *bla*$_{OXA}$ genes in clinical isolates is crucial, due to the potential for the spread and evolution of these genes in response to local selective pressures. Therefore, one of the main purposes of this thesis was the development and testing of a molecular PCR-based detection method for *bla*$_{KPC-3}$ and *bla*$_{OXA-48-like}$ genes as these genes are the most widespread in Portugal (126).

2.2.1. Oligonucleotide Primer Design

For this study, a set of primers was designed, which was carried out through three software, the OligoAnalyzer™ Tool, Oligo Explorer version 1.4 BETA, and CLC Sequence Viewer version 8.

The CLC Sequence Viewer software was used to visualize aligned nucleotide sequences corresponding to *bla*$_{KPC-3}$ and the two *bla*$_{OXA-48-like}$ genes and find conserved regions across different alleles. The primer design was carried out taking into account the genetic diversity observed for both genes, targeting conserved regions to cover as much as possible the entire open reading frames as to include allele-defining mutations (139). From this software, we create two sets of primers for this project. The first set of primers includes one Forward (Fw) and one Reverse (Rv) for each gene (*bla*$_{KPC-3}$ and *bla*$_{OXA-48-like}$), tested in a simplex PCR. While the second set of primers comprised one Rv and one internal primer (In) more central in the targeted region, for each gene, to test the amplification of both genes in a multiplex PCR reaction, along with the Fw primers of the first set (**Table 2.1**).

The OligoAnalyzer™ Tool software allowed us to calculate the T_m (melting temperature) of primers and estimate an appropriate annealing temperature based on the primer pair sequence, primer concentration, and DNA polymerase used in PCR. Additionally, this software also calculates the primer length, percentage of GC content, molecular weight, and extinction coefficient (140). Oligo Explorer version 1.4 BETA allowed us to determine primer properties like primer loops and primer dimers (141).

Table 2.1- Nucleotide sequence of each primer used for *bla*$_{KPC-3}$ and *bla*$_{OXA-48-like}$ amplifications.

Gene	1st Set of primers 5’- 3’	2nd Set of primers 5’- 3’
bla$_{KPC-3}$	Fw: ATGTCACTGTATCGCCGTCT	Rv: CTCCTCAGCGCGGTAACTTA
	Rv: TTATCACTGTATTGCACGGC	In: CCAAAGTCCTGTTCGAGTTT
bla$_{OXA-48-like}$	Fw: ATGCGTGTATTAGCCTTATC	Rv: GGTTCGCCCGTTTAAGATTA
	Rv: ATATCCATATTCATCGCAA	In: ATTCCGATAATCGATGCCAC

2.2.2. Optimization of PCR cycling conditions

All PCR reactions were performed with a final volume of 25 µL with NZYTaq II DNA polymerase (nzytech, Portugal), based on the manufacturer's protocol (142). For observation of PCR products, the gel preparation was performed at 1.5% of Agarose (nzytech, Portugal) in 1x Sodium Borate buffer (SB) and subsequently stained with GreenSafe Premium (NZYtech, Portugal) (143). The PCR conditions were optimized until we achieved the cycling conditions shown in **Table 2.2** and **Table 2.3**.

Table 2.2- Optimized cycling conditions with the first set of primers for *bla*$_{KPC-3}$ and *bla*$_{OXA-48-like}$ amplification in a simplex PCR.

Gene	Primers 5' – 3'	Denaturation	Cycles	Annealing	Final extension
bla$_{KPC-3}$	Fw: ATGTCACTGTATCGCCGTCT	95ºC 5 min	35X	95ºC 1 min	72ºC 10 min
				60ºC 1 min	
	Rv: TTATCACTGTATTGCACGGC			72ºC 1 min	
bla$_{OXA-48-like}$	Fw: ATGCGTGTATTAGCCTTATC	95ºC 5 min	35X	95ºC 1 min	72ºC 10 min
				57ºC 1 min	
	Rv: ATATCCATATTCATCGCAA			72ºC 1,30 min	

Table 2.3- Optimized cycling conditions with the second set of primers and the first set's Fw primers, for *bla*$_{KPC-3}$ and *bla*$_{OXA-48-like}$ genes amplification in a multiplex PCR.

Gene	Primers 5' – 3'	Denaturation	Cycles	Annealing	Final extension
bla$_{KPC-3}$	Fw: ATGTCACTGTATCGCCGTCT	95ºC 5 min	35X	95ºC 1 min	72ºC 10 min
	In: CCAAAGTCCTGTTCGAGTTT				
	Rv: CTCCTCAGCGCGGTAACTTA			57ºC 1 min	
bla$_{OXA-48-like}$	Fw: ATGCGTGTATTAGCCTTATC				
	In: ATTCCGATAATCGATGCCAC			72ºC 0,20 sec	
	Rv: GGTTCGCCCGTTTAAGATTA				

2.2.3. PCR detection of *bla*$_{KPC-3}$ and *bla*$_{OXA-48-like}$ genes in *K. pneumoniae* clinical isolates

The PCR molecular detection was tested on a larger sample set composed of different *Enterobacterales* clinical isolates previously characterized regarding phenotypic susceptibility to carbapenems and subjected to whole-genome sequencing. This project focused mainly on isolates of producing-carbapenemases, such as *Klebsiella pneumoniae*. A total of 52 clinical isolates, part of the strain collection of the Bacterial Pathogenomics and Drug Resistance Lab at iMed.ULisboa, were included (**Table 2.4**). For the simplex PCR detection, the expected size of the PCR products was 352bp for the *bla*$_{KPC}$ genes and 683bp for the *bla*$_{OXA}$ genes. While the expected size of the multiplex PCR products was 149bp for *bla*$_{KPC}$ and 150bp for *bla*$_{OXA}$.

Table 2.4- Description of all 52 clinical isolates of *Klebsiella pneumoniae*. **Abbreviations:** ND- not defined.

Strain ID	Microorganism	Year of isolation	Carbapenemase coding gene
5501	*Klebsiella pneumoniae*	2019	*bla*$_{OXA-181}$
5502	*Klebsiella pneumoniae*	2019	*bla*$_{KPC-3}$
5504	*Klebsiella pneumoniae*	2019	*bla*$_{KPC-3}$
5505	*Klebsiella pneumoniae*	2018	*bla*$_{KPC-3}$
5506	*Klebsiella pneumoniae*	2018	*bla*$_{OXA-181}$
5507	*Klebsiella oxytoca*	2018	ND
5508	*Klebsiella pneumoniae*	2018	*bla*$_{KPC-3}$
5509	*Klebsiella pneumoniae*	2018	*bla*$_{KPC-3}$
5510	*Klebsiella pneumoniae*	2018	ND
5511	*Klebsiella pneumoniae*	2019	*bla*$_{OXA-181}$
5512	*Escherichia coli*	2018	ND
5513	*Klebsiella pneumoniae*	2018	*bla*$_{KPC-3}$
5514	*Klebsiella pneumoniae*	2018	*bla*$_{KPC-3}$
5516	*Klebsiella pneumoniae*	2018	*bla*$_{KPC-3}$
5517	*Escherichia coli*	2018	ND
5518	*Klebsiella pneumoniae*	2019	*bla*$_{KPC-3}$
5519	*Klebsiella pneumoniae*	2019	*bla*$_{KPC-3}$
5520	*Klebsiella pneumoniae*	2019	*bla*$_{KPC-3}$
5521	*Klebsiella pneumoniae*	2019	*bla*$_{KPC-3}$
5522	*Klebsiella pneumoniae*	2019	*bla*$_{KPC-3}$
5524	*Klebsiella pneumoniae*	2019	*bla*$_{KPC-3}$
5525	*Klebsiella pneumoniae*	2019	*bla*$_{OXA-181}$
5526	*Klebsiella pneumoniae*	2019	*bla*$_{KPC-3}$
5527	*Salmonella enteritidis*	2019	ND
5528	*Klebsiella pneumoniae*	2019	ND
4845	*Klebsiella pneumoniae*	2013	ND
4852	*Klebsiella pneumoniae*	2013	*bla*$_{KPC-3}$
4855	*Klebsiella pneumoniae*	2013	ND
4856	*Klebsiella pneumoniae*	2014	ND
4857	*Klebsiella pneumoniae*	2014	ND
4859	*Klebsiella pneumoniae*	2014	ND
4860	*Klebsiella pneumoniae*	2014	ND
4861	*Klebsiella pneumoniae*	2014	ND
4862	*Klebsiella pneumoniae*	2014	ND
4864	*Klebsiella pneumoniae*	2015	ND
4865	*Klebsiella pneumoniae*	2015	ND
4867	*Klebsiella pneumoniae*	2015	ND
4869	*Klebsiella pneumoniae*	2011	ND
4871	*Klebsiella pneumoniae*	2015	ND
4874	*Klebsiella pneumoniae*	2015	ND
4878	*Klebsiella pneumoniae*	2015	ND
4879	*Klebsiella pneumoniae*	2015	ND
4880	*Klebsiella pneumoniae*	2015	ND
4882	*Klebsiella pneumoniae*	2016	*bla*$_{GES-5}$, *bla*$_{KPC-3}$
4884	*Klebsiella pneumoniae*	2016	*bla*$_{GES-5}$, *bla*$_{KPC-3}$

4885	*Klebsiella pneumoniae*	2016	*bla*$_{GES-5}$, *bla*$_{KPC-3}$
4886	*Klebsiella pneumoniae*	2016	*bla*$_{GES-5}$, *bla*$_{KPC-3}$
4887	*Klebsiella pneumoniae*	2016	*bla*$_{GES-5}$, *bla*$_{KPC-3}$
4958	*Klebsiella pneumoniae*	2011	ND
3760	*Klebsiella pneumoniae*	2010	*bla*$_{KPC-3}$
1000	*Klebsiella pneumoniae*	2004	ND
1001	*Klebsiella pneumoniae*	2004	ND

2.2.3.1. Culture and DNA extraction

Bacterial culture was carried out from frozen stocks stored in Brain Heart Infusion (BHI) (Oxoid, England) supplemented with 15% glycerol at -80 ºC in Drigalski Lactose Agar medium (Frilabo, Italy). These bacterial cultures were left overnight at 36 ºC (Cellstar).

Crude DNA extracts were obtained by the boiling-centrifugation method. Briefly, one loopful of bacterial growth was resuspended in 1 mL of 1x TE buffer (10 mM Tris-HCl, 1mM EDTA•Na2, pH 8.0) and lysed for 10 minutes in a boiling water bath at 95 °C-100 ºC (Grant, England). Cellular debris were removed by centrifugation (Eppendorf AG, Hamburg) at 16000 rpm for 10 minutes and the supernatant was collected and used as template DNA for downstream PCR reactions.

2.3. Ceftazidime-avibactam (CZA) antimicrobial susceptibility test in *Klebsiella pneumoniae* isolates

Ceftazidime-avibactam (CZA) minimum Inhibitory concentration (MIC) was determined for selected isolates (**Table 2.5**) sing the broth microdilution method in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (144–146).

Table 2.5- Description of all 5 *Klebsiella pneumoniae* strains tested for susceptibility to CZA.

Strain ID	Microorganism	Carbapenemase coding gene known
5506	*Klebsiella pneumoniae*	*bla*$_{OXA-181}$
5511	*Klebsiella pneumoniae*	*bla*$_{OXA-181}$
4887	*Klebsiella pneumoniae*	*bla*$_{GES-5}$, *bla*$_{KPC-3}$
3734	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$
3826	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$

The MIC determination is the concentration at which no bacterial growth can be observed by the naked eye as a result of the antibiotic action. The preparation of the CZA combination was performed according to the EUCAST guidelines with a constant avibactam concentration of 4 mg/L. The MIC values obtained were translated to resistant or susceptible phenotype according to EUCAST breakpoints: *Enterobacterales* susceptible isolates display a MIC ≤ 8 mg/L and resistant isolates a MIC > 8 mg/L. In *Enterobacterales* bacteria for the CZA antibiotic, no Area of Technical Uncertainty (ATU) is defined (146). Also, the EUCAST does not recommend the use of a specific quality control (QC) strain, thus in this project, we used the *Pseudomonas aeruginosa* ATCC 27853 as the QC (MIC range = 0.5–4 mg/L) (145).

3. Results and Discussion

The present study sought to investigate and characterize the genetic diversity and phylogenetic relationships between different alleles of KPC and OXA β-lactamase coding genes, along with its taxonomical dispersion and association with distinct bacterial families. For this purpose, the reference nucleotide sequence for each KPC and OXA allele was retrieved from NCBI Reference Gene Catalog for antimicrobial resistance genes. This included a total of 88 and 428 *bla*$_{KPC}$ and *bla*$_{OXA}$ alleles, respectively. As well as a total of 1232 *bla*$_{KPC}$ and 2204 *bla*$_{OXA}$ hit accessions (**Table 3.1**). Below, the phylogenetic relationship between different alleles, their diversity, correlation with carbapenemase activity, and taxonomical spread are explored.

Table 3.1- List of all allele sequences of *bla*$_{KPC}$ and *bla*$_{OXA}$ genes, as well as all hit accessions for each allele. **Abbreviations:** Not carb- no carbapenemase activity; Carb- carbapenemase activity. **Data from:** (100,115).

	***bla*$_{KPC}$ allele sequences**		***bla*$_{OXA}$ allele sequences**		***bla*$_{KPC}$ hit sequences**	***bla*$_{OXA}$ hit sequences**
	Not carb	*Carb*	*Not carb*	*Carb*	1232	2204
	15	73	188	240		
Total	88		428		3436	

3.1. Diversity and taxonomical distribution of KPC and OXA coding genes

3.1.1. KPC coding genes

The study includes a total of 88 KPC coding genes, each corresponding to a different allele, 73 of which correspond to carbapenemase coding genes according to the available metadata that is curated by the NCBI (**Table3.1**). Phylogenetic reconstruction using the sequence data obtained and upon multiple sequence alignment revealed a topological structure composed mainly of three phylogenetic groups including KPC-2, KPC-3, and a third containing KPC-4 and KPC-5. The first and second KPC groups were then and hereby designated as KPC-2-like and KPC-3-like, respectively. According to the literature, KPC-5 and KPC-6 were intermediators between KPC-2 and KPC-4 (110,147), implying that KPC-4 is a variant of KPC-5, and thus the third group was herein named KPC-5-like (**Table 3.2**).

Also, according to the literature mutations in the *bla*$_{KPC-2}$ structural gene resulted in many variants, such as KPC-3 (148), KPC-4, KPC-5, and KPC-6 (147).

Table 3.2- List of all alleles of each KPC group (KPC-2-like, KPC-3-like, KPC-5-like), and the number of alleles coding for carbapenemase enzymes.

KPC group	No. of alleles	No. of alleles coding carbapenemase enzymes
KPC-2-like	37	31
KPC-3-like	22	18
KPC-5-like	29	21

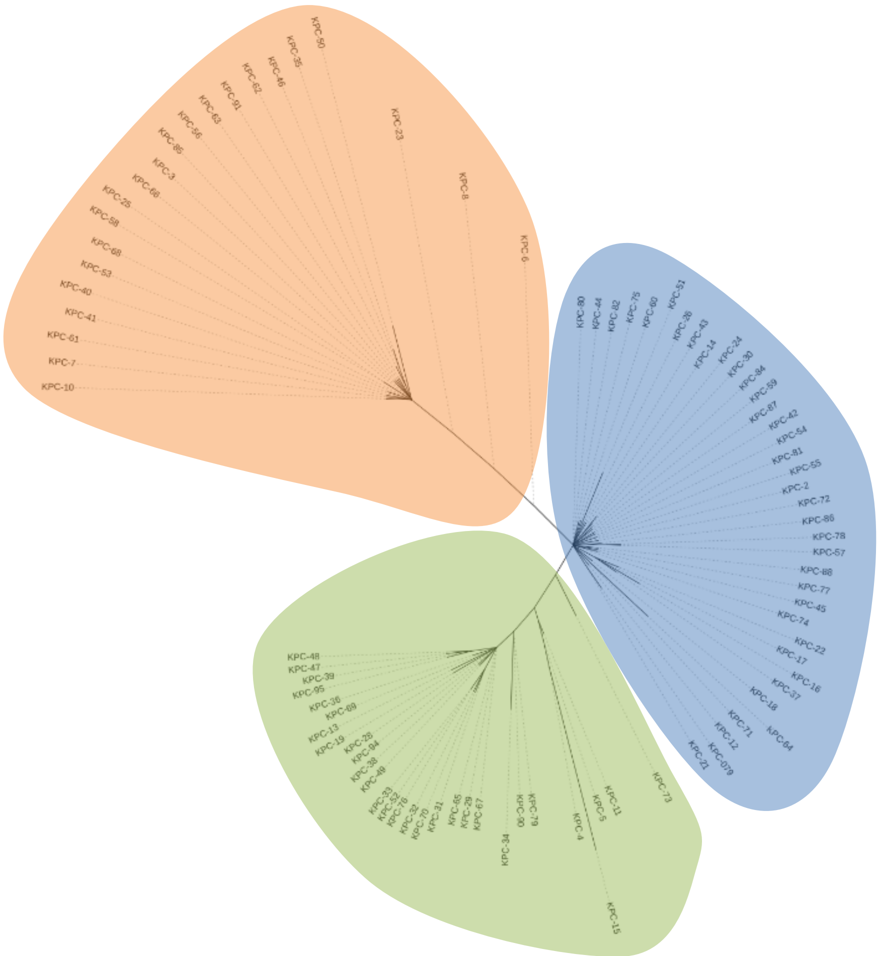

Figure 3.1- Phylogenetic tree of groups KPC-2-like, KPC-3-like and KPC-5-like. Blue shape represents the KPC-2-like group, orange shape the KPC-3-like and green shape the KPC-5-like. **Created using:** (134).

It is noteworthy the polytomic phylogenetic structure characterizing all three KPC groups suggesting common descent and radiation from a common ancestor (**Figure 3.1**).

Interestingly, KPC-4 and KPC-5 comprise the same group (KPC-5-like), which is consistent with the fact that KPC-5 was an intermediator to KPC-4. Likewise, KPC-6 was expected to harbor either the KPC-5-like group or the KPC-2-like group, since KPC-6 was an intermediator to KPC-4 and is a variant of KPC-2. Nonetheless, the presence of KPC-6 was detected in the KCP-3-like group, possibly justified by a divergent evolution of this gene.

3.1.1.1. Taxonomical dispersion of *bla*$_{KPC}$ genes

Next, we explored the taxonomical dispersion of the 88 *bla*$_{KPC}$ genes by performing a programmatic tblastn analysis on the entire nucleotide sequence database of the NCBI, using the protein sequences of each allele. The data obtained were analyzed regarding the genus taxon associated with each hit accession. A total of 17 genera were found associated with 1232 *bla*$_{KPC}$ alleles with special prevalence for the *Klebsiella* genus, found to harbor at least 853 different *bla*$_{KPC}$ alleles, followed by *Escherichia* (n=110) and *Citrobacter* (n=68) **(Figure 3.2** and **Figure 3.3**; **Table 3.3**).

From a gene dissemination viewpoint, we stress the importance of *bla*$_{KPC-2}$ as a highly disseminated enzyme whose coding gene is herein found across 17 genera, particularly in *Klebsiella* (n=572/888), and *bla*$_{KPC-3}$ which was detected across 7 out of 17 genera (*Acinetobacter*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Raoultella*, *Serratia*, and *Klebsiella*), which is consistent with previous reports (105,128). The *bla*$_{KPC-4}$, *bla*$_{KPC-5,}$ and *bla*$_{KPC-6}$ were present in three different genera, highlighting their potential for cross-genera mobilization (**Figure 3.3**; **Table 3.3**).

Table 3.3- Description of the three prevalent genera reported in the 1232 *bla*$_{KPC}$ hit accessions obtained upon programmatic tblastn search and analysis, and the number of KPC-2 and KPC-3 alleles among each genus.

No. of genera	Prevalent genus	No. of KPC-2 alleles	No. of KPC-3 alleles	Total
17	*Klebsiella*	572	108	853
	Escherichia	69	23	110
	Citrobacter	50	16	68
	Total	888	173	

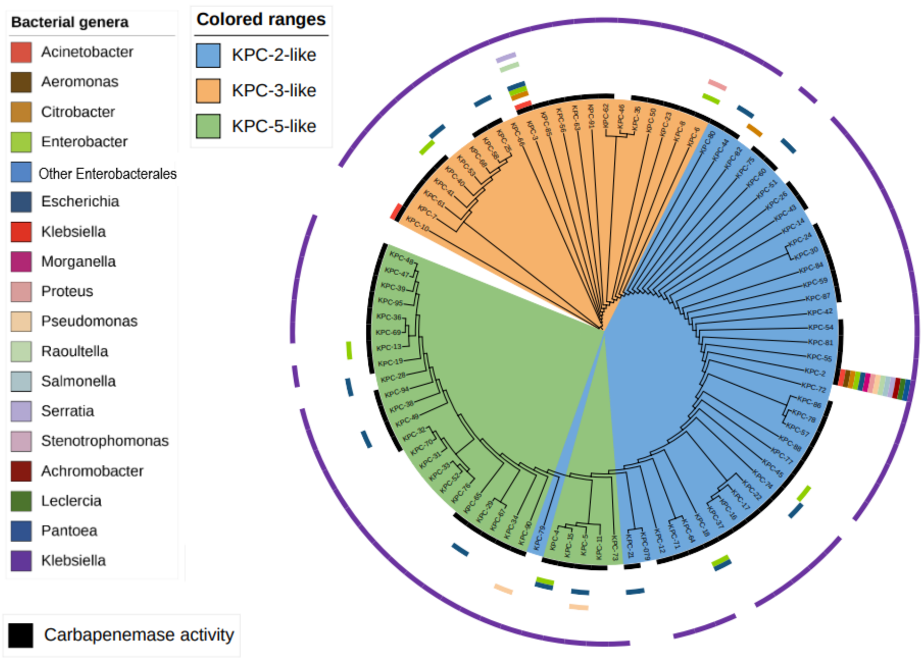

Figure 3.2- Phylogenetic tree of all 88 *bla*KPC genes, associated with 17 distinct bacterial genera. The presence of a colored strip on each allele represents the bacterial genera with which that allele is associated. Each strip color represents a genus, listed in the Bacterial genera legend. The black strip represents all the *bla*KPC alleles with carbapenemase activity, and the colored ranges all three KPC groups. **Created using:** (149).

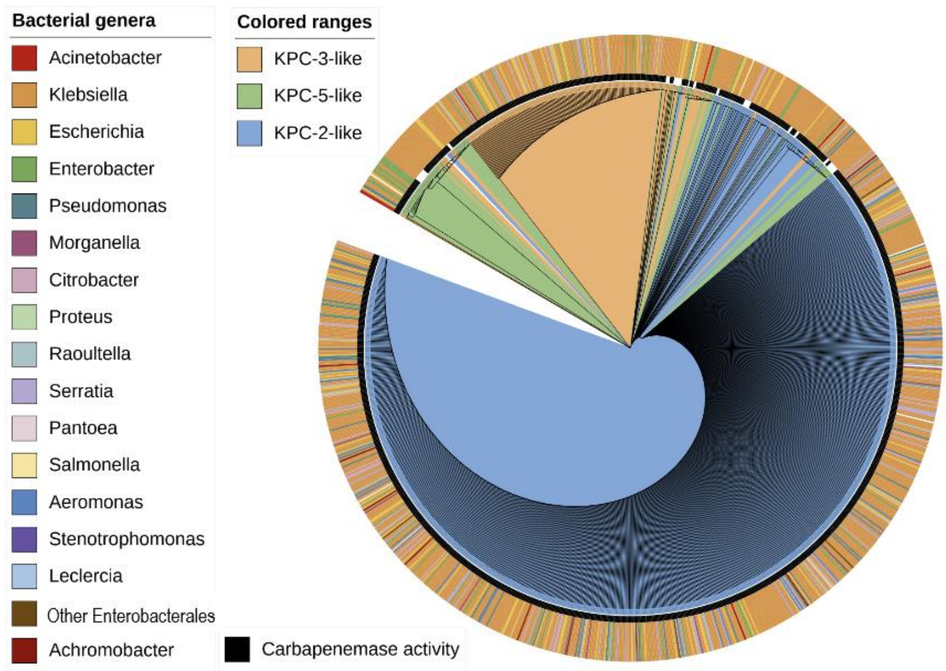

Figure 3.3- Phylogenetic tree of all 1232 *bla*KPC alleles reported in 17 bacterial genera. The presence of a colored strip on each allele represents the bacterial genera with which that allele is associated. Each strip color represents a genus, listed in the Bacterial genera legend. The black strip represents all the *bla*KPC alleles with carbapenemase activity, and the colored ranges all three KPC groups. **Created using:** (149).

According to **Figure 3.2,** KPC-79 is the only KPC-2-like allele located phylogenetically in the middle of the KPC-5-like group, suggesting a divergent evolution of this allele towards its KPC group, establishing a closer phylogenetic relationship with some KPC-5-like alleles. A recent article reported that *bla*$_{KPC-79}$ is a variant derived from *bla*$_{KPC-2}$ mutations (150), which is in line with our results that state that KPC-79 is harbored in the KPC-2-like group. However, KPC-79 showed a closest phylogenetic relationship with some KPC-5-like alleles, which could be explained by previous findings by Q. Shi et al (2022), that report a carbapenemase KPC-79 coding gene with a 7-amino-acid tandem repeat between two amino acids, compared with KPC-2, further evolved into KPC-76 through a substitution during ceftazidime-avibactam exposure (151). Noteworthy the KPC-76 is harbored in the KPC-5-like group (**Figure 3.1**). Therefore, this possible divergent evolution from the KPC-79 could be an intermediate step between KPC-2 and KPC-5-like variants. Also interesting, the same article, reports that the carbapenemase KPC-79 was detected on IncFII/IncR-type plasmids (151), which were already described in previous studies as associated with a rapid evolution of carbapenemase resistance through horizontal gene transfer events (152,153).

On the other hand, in **Figure 3.3**, all KPC groups presented some close phylogenetic relationships to alleles from different groups, including the relationship between KPC-79 and KPC-5-like alleles. This highlights the KPC capacity of cross-gene transfer events between these genes over the years.

3.1.1.2. Monophyletic integrity and convergent evolution of *bla*$_{KPC}$ genes

Having determined the taxonomical dispersion patterns of *bla*$_{KPC}$ genes, we explored the evolutionary trajectories across all *bla*$_{KPC}$ genes integrated into a new reconstructed phylogenetic scenario that encompasses all *bla*$_{KPC}$ gene hits identified in the previous analysis (n=1232). The phylogenetic tree obtained did not show a structure compatible with the taxonomical origin of the identified gene sequences. These findings are compatible with a scenario in which dissemination across different genera is dominated by multiple and constant events of lateral gene transfer between different taxa **(Figure 3.3**).

Individually gene analyses evaluating all hits of each gene revealed that the most common KPC coding genes (KPC-2 and KPC-3) did not disclose a monophyletic structure, whereas the third most common, KPC-4, KPC-5, and KPC-6, did. Likewise, all the other genes were individually analyzed and all data was collected by group (**Table 3.4**).

Table 3.4- Monophyletic description of all KPC condign genes of each KPC-like group (KPC-2-like, KPC-3-like, and KPC-5-like).

KPC-like groups	No. of KPC alleles with monophyletic structure	No. of KPC alleles without monophyletic structure	Total of KPC alleles
KPC-2-like	29	8	37
KPC-3-like	15	7	22
KPC-5-like	21	8	29

A closer look at **Figure 3.3**, demonstrates one main clade with two branches, where one of the branches has all the KPC-10 alleles (KPC-10_GQ140348 and KPC-10_NG 049243) isolated, and the second branch extends for another clade with two branches, one of which embraces 1204 alleles (grey shape in (**Figure 3.4**), and the other 26 alleles. Interestingly, all alleles of the most common KPC coding genes (KPC-2 and KPC-3) are harbored in the grey shape along with KPC-6, while two of the third most prevalent (KPC-4 and KPC-5) are present among the 26 alleles. Revealing a divergent evolution of KPC-4 and KPC-5 from KPC-2, their KPC ancestor. Noteworthy, all the KPC-10 alleles are isolated in this phylogenetic structure, implying a divergent evolution of this coding gene towards its KPC ancestors, that according to literature may be KPC-3 or KPC-5 (147).

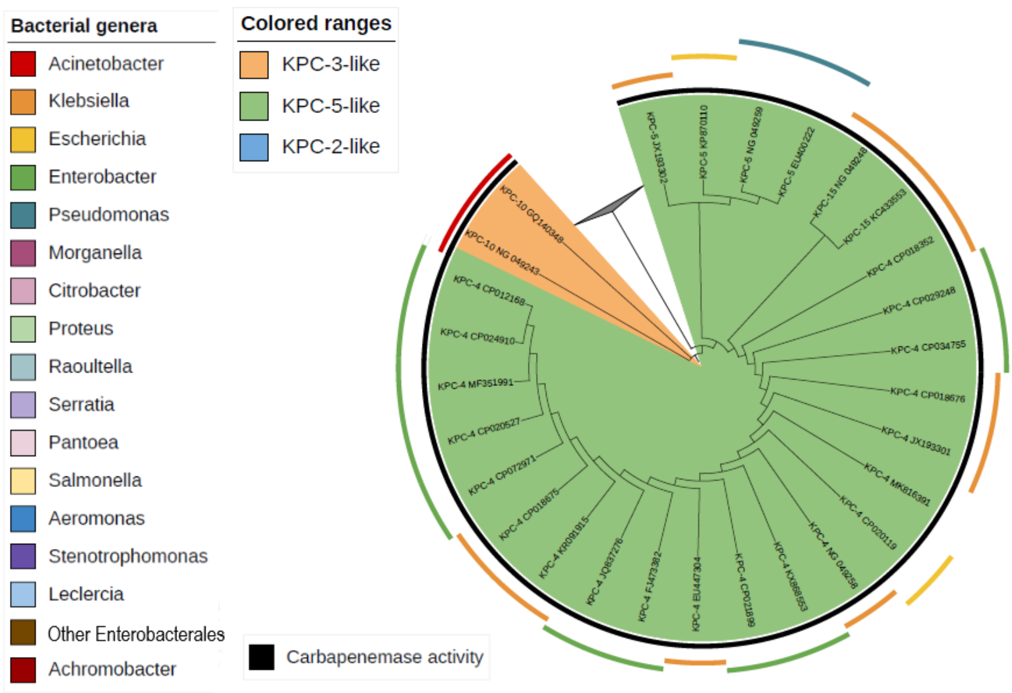

Figure 3.4- Phylogenetic tree of all 1232 *bla*$_{KPC}$ allele sequences, associated with 17 distinct bacterial genera. The presence of a colored strip on each allele represents the bacterial genera with which that allele is associated. Each strip color represents a genus, listed in Bacterial genera legend. The black strip represents all the *bla*$_{KPC}$ alleles with carbapenemase activity, and the colored ranges all the three KPC groups. The grey shape represents the collapse clade, harboring 1204 alleles. **Created using:** (149).

3.1.1.3. Genetic context of *bla*$_{KPC}$ genes

It is known that *bla*$_{KPC}$ genes are frequently inserted in the transposable element Tn*4401* and that this has led to a worldwide dissemination of *bla*$_{KPC}$ genes. To investigate the prevalence and association between different isoforms and alleles, we attempted to characterize the genetic context of each *bla*$_{KPC}$ gene identified in the blast analysis regarding whether or not these genes are associated with this transposable element. We also attempted to characterize the Tn*4401* isoform and seek new isoforms.

With these analyses we were able to determine the Tn*4401* isoforms of 450 *bla*$_{KPC}$ alleles, enabling the identification of three isoforms in this dataset: Tn*4401a* (n=200; 44,4%); Tn*4401b* (n=224; 49,8%); Tn*4401d* (n=26; 5,8%) (**Table 3.5**; **Supplementary Table S 6.1**). *Klebsiella pneumoniae* was found to be the bacterial genus with the most reports of Tn*4401* isoforms (n=276) corresponding to approximately 61% of the *bla*$_{KPC}$ alleles total, followed by *Escherichia* (n=44) with nearly 10%, and *Citrobacter* (n=40) with 9%.

Table 3.5- Description of the three most prevalent bacterial genera reported with a Tn*4401* transposon, and the number of Tn*4401* isoforms identify in each pathogen.

Tn*4401* isoforms	**Klebsiella**	**Escherichia**	**Citrobacter**	**Total**
Tn*4401a*	158 (57%)	18 (41%)	13 (33%)	200
Tn*4401b*	102 (37%)	21 (48%)	27 (68%)	224
Tn*4401d*	16 (6%)	5 (11%)	0 (0%)	26
Total	276 (100%)	44 (100%)	40 (100%)	

Interestingly, the Tn*4401a* and Tn*4401b* revealed similar percentages of prevalence in the *Escherichia* genus (41% and 48%, respectively), but not in *Klebsiella* or *Citrobacter*.

The distribution of Tn*4401* transposon isoforms across the *bla*$_{KPC}$ phylogenetic tree was also examined (**Figure 3.5)**, where Tn*4401a* and Tn*4401b* transposons are shown as the most widespread, as stated in the literature (86,88–91). Interestingly, Tn*4401d* appears to be restricted mainly among *bla*$_{KPC\text{-}3\text{-}like}$ genes, as described in previous studies (93,94,155,156), albeit in our findings it has been observed among a specific *bla*$_{KPC\text{-}5\text{-}like}$ subbranch (KPC-31_CP043048). To our knowledge, there is only one report of a KPC-31 associated with accession no. CP043048, which reports that the *bla*$_{KPC\text{-}31}$ gene was discovered harbored in the pKLP268-2 plasmid from a *K. pneumoniae* strain, isolated in 2019 from a gull fecal sample, in Lisbon, Portugal (156). Therefore, more research regarding the KPC-31_CP043048 of our study and its possible inclusion in the pKLP268-2 plasmid may be necessary.

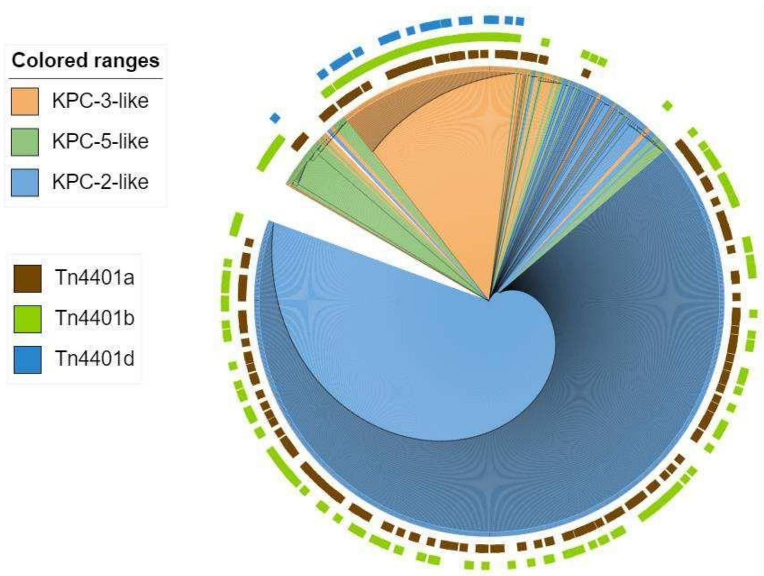

Figure 3.5- Phylogenetic tree with all 450 *bla*$_{KPC}$ hit accessions identified in Tn*4401* isoforms (a, b and d). The presence of a colored strip on each hit represents the Tn*4401* isoform with which that allele is associated. The colored ranges represent the KPC-like group, listed in the legend. **Created using:** (149).

3.1.2. <u>OXA coding genes</u>

Analyses of OXA coding genes were also carried out in this study, with a total of 428 *bla*$_{OXA}$ alleles, 240 of which were identified as carbapenemase producers. These *bla*$_{OXA}$ alleles were grouped by all the five OXA-like groups existing in NCBI metadata (OXA-23-like, OXA-24/40-like, OXA-48-like, OXA-51-like, and OXA-58-like), and a phylogenetic tree was generated (**Table 3.6**; **Figure 3.6**).

Our findings revealed a phylogenetic tree divided into five groups, as expected, where OXA-51-like is the group with the higher number of alleles, followed by OXA-23-like and OXA-48-like with a phylogenetic congruence with the same number of alleles (n=41). Also, the group with the lower number of alleles (OXA-58-like) was the only in which all alleles have carbapenemase activity.

Table 3.6- Distribution of OXA alleles by group (OXA-23-like, OXA-24/40-like, OXA-48-like, OXA-51-like, OXA-58-like), and the number of alleles coding for carbapenemase enzymes.

OXA group	No. of alleles	No. of alleles coding carbapenemase enzymes
OXA-23-like	41	26
OXA-24/40-like	10	8

OXA-48-like	41	17
OXA-51-like	329	182
OXA-58-like	7	7

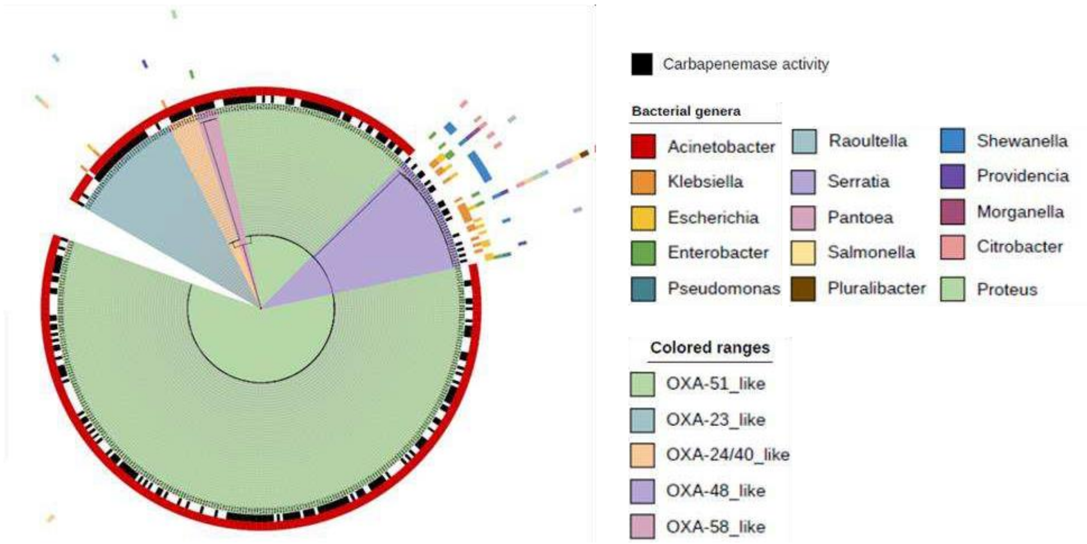

Figure 3.6- Phylogenetic tree of all 428 *bla*$_{OXA}$ allele sequences, associated with 15 distinct bacterial genera. The presence of a colored strip on each allele represents the bacterial genera with which that allele is associated. Each strip color represents a genus, listed in Bacterial genera legend. The black strip represents all the *bla*$_{OXA}$ alleles with carbapenemase activity, and the colored ranges all the OXA groups. **Created using:** (149).

3.1.2.1. Taxonomical dispersion of *bla*$_{OXA}$ genes

The taxonomical dispersion of *bla*$_{OXA}$ genes was subsequently analyzed using a similar approach as described for the *bla*$_{KPC}$ genes, resulting in a total of 2204 *bla*$_{OXA}$ hit accessions obtained. These analyses were carried out through 15 different genera, with special prevalence for *Acinetobacter*, found to harbor at least 1639 of the hits obtained, followed by *Klebsiella* (n=326) and *Escherichia* (n=208) (**Figure 3.7**; **Table 3.7**).

These results reveal OXA-48-like and OXA-51-like as the most prevalent groups with 648 and 1002 alleles, respectively. The OXA-23-like and OXA-51-like are the most prevalent among the *Acinetobacter* genus, which is in line with previous reports (117–121), and the OXA-48-like the most prevalent among *Enterobacterales*, as reported in the literature (116).

Table 3.7- Description of the three prevalent genera associated with the 2204 *bla*$_{OXA}$ hit accessions, and their number among the OXA-23-like, OXA-48-like, and OXA-51-like groups.

Top prevalent genus	No. of OXA-23-like alleles	No. of OXA-48-like alleles	No. of OXA-51-like alleles	Total
Acinetobacter	433	0	1001	1639
Klebsiella	3	322	0	326
Escherichia	3	205	0	208
Total	448	648	1002	

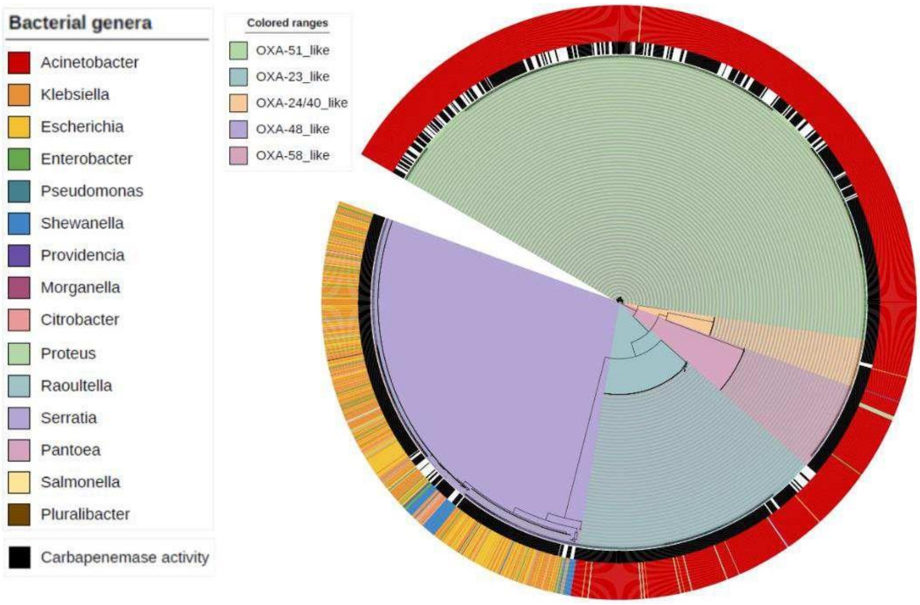

Figure 3.7- Phylogenetic tree of all 2204 *bla*OXA hit accessions reported in 15 bacterial genera. The presence of a colored strip on each hit represents the bacterial genera with which that allele is associated. Each strip color represents a genus, listed in Bacterial genera legend. The black strip represents all the *bla*OXA alleles with carbapenemase activity, and the colored ranges all the OXA groups. **Created using:** (149).

A closer look into **Figure 3.6** and **Figure 3.7,** revealed a highly disseminated OXA-48-like group, whose coding gene is herein found across 13 different genera, especially in *Klebsiella* (n=322/326). Among this group, *bla*OXA-48 and *bla*OXA-181 were the most disseminated genes, reported in 13 and 7 genera, respectively (**Figure 3.8**; **Figure 3.9**), as described in previous studies (80,157–159).

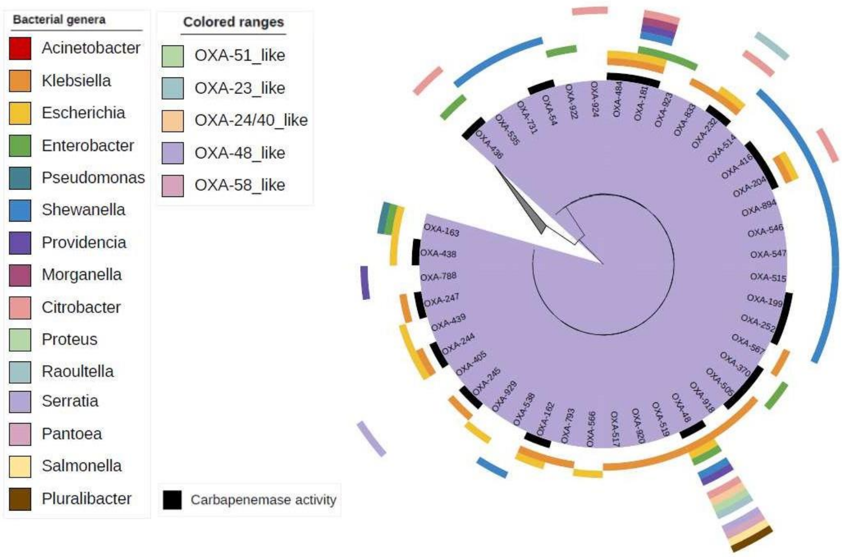

Figure 3.8- A closer look into Figure 3.6 phylogenetic tree, of OXA-48-like group alleles, with 41 *bla*OXA-48-like alleles and 15 bacterial genera. The presence of a colored strip on each allele represents the bacterial genera with which that allele is associated. Each strip color represents a genus, listed in Bacterial genera legend. The black strip represents all the *bla*OXA-48-like alleles with carbapenemase activity, and the colored ranges all the OXA groups. The gray shape represents the collapse clades of the other groups. **Created using:** (149).

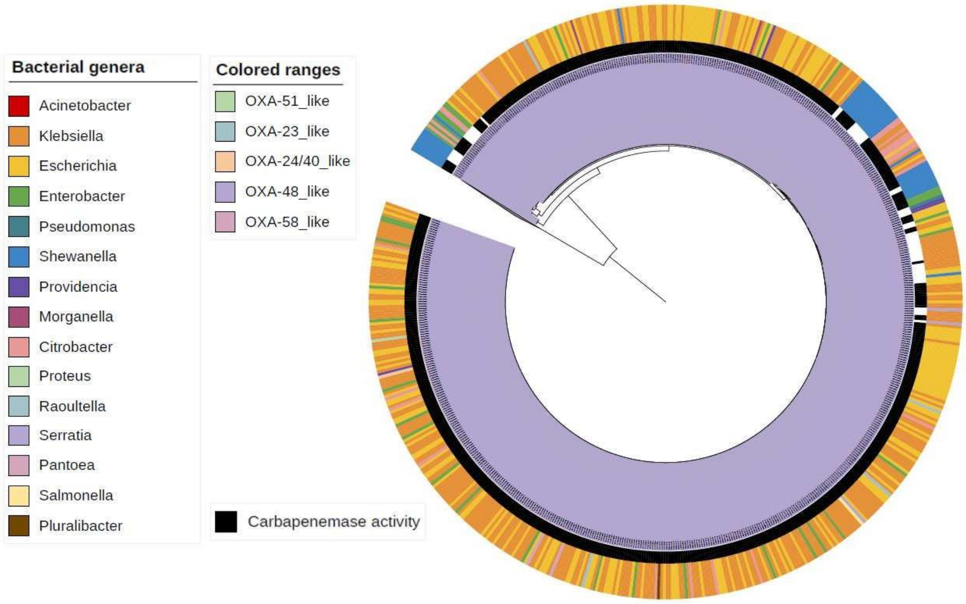

Figure 3.9- A closer look into Figure 3.7 phylogenetic tree, of OXA-48-like group hit accessions, with 648 *bla*$_{\text{OXA-48-like}}$ alleles and 15 bacterial genera. The presence of a colored strip on each hit represents the bacterial genera with which that allele is associated. Each strip color represents a genus, listed in Bacterial genera legend. The black strip represents all the *bla*$_{\text{OXA-48-like}}$ alleles with carbapenemase activity, and the colored ranges all the OXA groups. **Created using:** (149).

Among the OXA-51-like alleles in **Figure3.7**, all the hit accessions were found across the *Acinetobacter* genus (n=1001/1002), with an exception. A *bla*$_{\text{OXA-51}}$ gene (MK847907) was detected in a *Salmonella* isolate with carbapenemase activity **(Figure 3.10**). To our knowledge, to date the OXA-51 coding gene was only found in *A. baumannii*, *K. pneumoniae* (160), *Enterobacter cloacae*, and *Escherichia coli* (161) isolates.

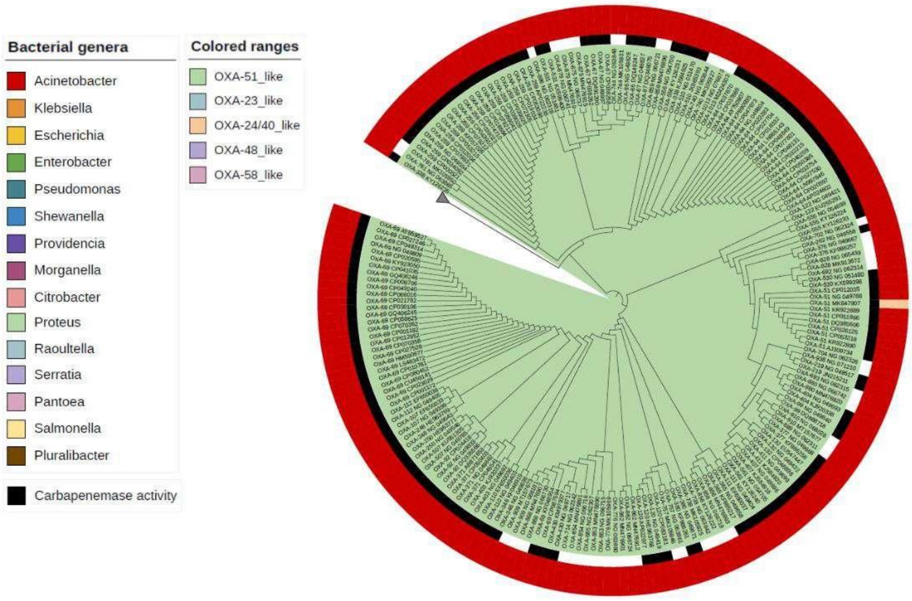

Figure 3.10- A closer look into Figure 3.7 phylogenetic tree, of OXA-51-like group hit accessions, with 1002 *bla*$_{\text{OXA-51-like}}$ alleles and 15 bacterial genera. The presence of a colored strip on each hit represents the bacterial genera with which that allele is associated. Each strip color represents a genus, listed in Bacterial genera legend. The black strip represents all the *bla*$_{\text{OXA-51-like}}$ alleles with carbapenemase activity, and the colored ranges all the OXA groups. The gray shape represents the collapse clades of the rest of the phylogenetic tree. **Created using:** (149).

Interestingly, the OXA-58-like group uncovered what appears to be a significant outlier in this group (**Figure 3.11**). Although this group is mainly found across *Acinetobacter*, a monophyletic group of five *bla*$_{OXA-58}$ alleles were found in *Proteus* genus isolates (KU871397; MK086028; MK533136; KX668205; KU871396), possibly representing a distinct sub-branch resulting from the lateral dissemination of *bla*$_{OXA-58}$ genes between these two genera followed by clonal dissemination. Noticeably, all five *bla*$_{OXA-58}$ alleles with carbapenemase activity harbored in the same clade, suggesting a common ancestor.

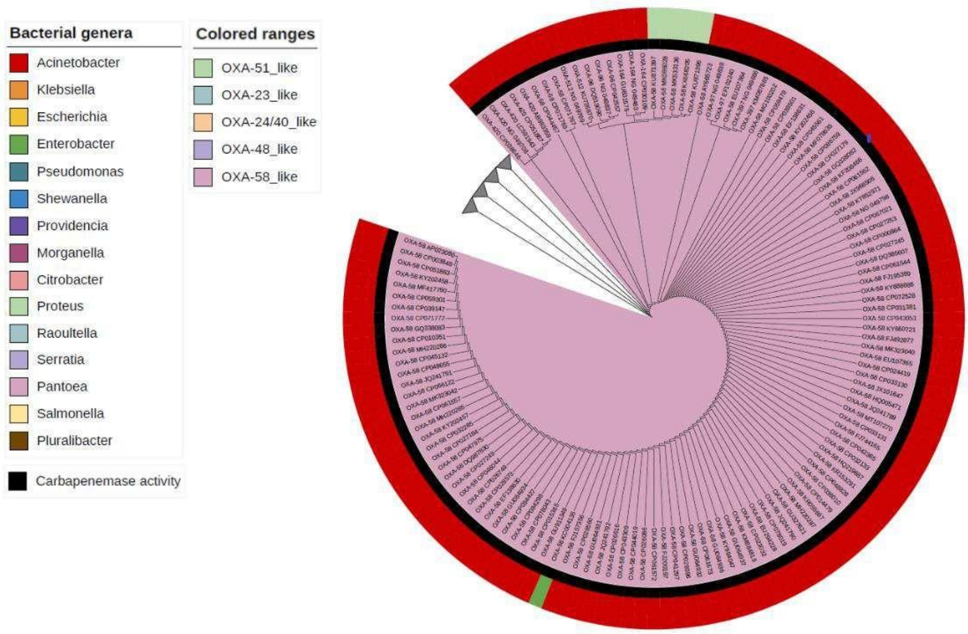

Figure 3.11- A closer look into Figure 3.7 phylogenetic tree, of OXA-58-like group hit accessions, with 138 *bla*$_{OXA-58-like}$ alleles and 15 bacterial genera. The presence of a colored strip on each hit represents the bacterial genera with which that allele is associated. Each strip color represents a genus, listed in Bacterial genera legend. The black strip represents all the *bla*$_{OXA-58-like}$ alleles with carbapenemase activity, and the colored ranges all the OXA groups. The gray shapes represent the collapse clades of the rest of the phylogenetic tree. **Created using:** (149).

According to previous studies, the *bla*$_{OXA-58}$ allele accession no. MK086028 was found in a *Proteus mirabilis* strain from 2017, from the blood of a 37-year-old hematological patient in a Poland hospital (162). The *Proteus* isolate with *bla*$_{OXA-58}$ allele accession no. KX668205 was isolated from blood culture and subsequently from peritoneal fluid specimens from a 53-year-old man in Belgium, in 2015 (163). Meanwhile, accession no. MK533136 *bla*$_{OXA-58}$ allele was also discovered in Belgium, in 2013 from a patient with sample collection information not available (164). Lastly, accession no. KU871397 and KU871396 *bla*$_{OXA-58}$ alleles were isolated from *Proteus* strains in Germany, between 2013 and 2015 (165). These studies emphasize the transmission and dissemination of this group of five *bla*$_{OXA-58}$ genes across different countries from Europe.

Also, highlighted in this figure **(Figure 3.11)**, is an OXA-58 (KC004135) with expression in an *Enterobacter* genus, already described in the literature, reported from an African hospital (161).

3.1.2.2. Monophyletic integrity and convergent evolution of *bla*$_{OXA}$ genes

The study of evolutionary trajectories across all *bla*$_{OXA}$ genes were also explored. All 2204 *bla*$_{OXA}$ hit accessions were analyzed and each gene was characterized according to its monophyletic integrity. These analyses were performed by group, and the number of OXA coding genes displaying a monophyletic structure is described in **Table 3.8**.

Analyses of all hits of each gene revealed that all *bla*$_{OXA-23}$ alleles, as well as all *bla*$_{OXA-48}$, *bla*$_{OXA-58,}$ and *bla*$_{OXA-181}$ alleles, did not have a monophyletic structure. All *bla*$_{OXA-24}$ and *bla*$_{OXA-51}$ alleles, on the other hand, showed monophyletic integrity (**Figure 3.7**).

Table 3.8- Monophyletic description of all OXA condign genes of each OXA-like group (OXA-23-like, OXA-24/40-like, OXA-48-like, OXA-51-like and OXA-58-like).

OXA-like groups	No. of OXA coding genes with monophyletic structure	No. of OXA coding genes without monophyletic structure	Total of OXA alleles
OXA-23-like	40	1	41
OXA-24/40-like	10	0	10
OXA-48-like	35	6	41
OXA-51-like	308	21	329
OXA-58-like	6	1	7

A close examination of **Figure 3.7** reveals one main clade with two branches, one extending for another clade harboring the OXA-23-like, OXA-24/40-like, OXA-48-like, and OXA-58-like groups, and the other branch with OXA-51-like group isolated. It is also noticeable a monophyletic relationship between OXA-48-like and OXA-23-like groups, and between OXA-24/40-like and OXA-58-like. These evolutions and relationships between OXA groups have not yet been clearly reported, implying that more research is required.

3.2. Molecular detection of *bla*$_{KPC-3}$ and *bla*$_{OXA-48-like}$

The second part of the study encompassed the design and test of a rapid PCR-based method enabling the detection of *bla*$_{KPC-3}$ and *bla*$_{OXA-48-like}$ genes (*bla*$_{OXA-48}$ and *bla*$_{OXA-181}$). The designing of this method benefited from the previous findings on the allelic diversity of *bla*$_{KPC-3}$ and *bla*$_{OXA-48-like}$ regarding primer design and targeted regions.

3.2.1. PCR detection of *bla*$_{KPC-3}$ and *bla*$_{OXA-48-like}$ genes in *K. pneumoniae* clinical isolates

The PCR molecular detection was tested on a set of 52 *Enterobacterales* clinical isolates composed mainly of *K. pneumoniae*, previously characterized regarding phenotypic susceptibility to carbapenems and subjected to whole-genome sequencing, and belonging to the strain collection of the Bacterial Pathogenomics and Drug Resistance Lab at iMed.ULisboa.

Through the first set of primers, we could confirm the presence of *bla*$_{KPC-3}$ and *bla*$_{OXA-181}$ genes in 20 and 4 isolates respectively (**Table 3.9**, **Figure 3.12**). Two out of the 52 isolates tested (5518 and 5519) failed to produce *bla*$_{KPC}$ amplicons despite previous confirmation by whole-genome sequencing. The two latter isolates consistently produced negative results by multiplex and simplex PCR highlighting the need for further studies to elucidate such discrepancy which at this point remains unknown.

Table 3.9- Description of all 52 strains, with their known carbapenemase coding genes, and the identified carbapenemase coding genes for each strain.

Strain isolate ID	Microorganism	Carbapenemase coding gene	Simplex PCR detection	
			bla $_{KPC-3}$	***bla*** $_{OXA-181}$
5501	*Klebsiella pneumoniae*	*bla*$_{OXA-181}$	-	+
5502	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
5504	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
5505	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
5506	*Klebsiella pneumoniae*	*bla*$_{OXA-181}$	-	+
5507	*Klebsiella oxytoca*	-	-	-
5508	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
5509	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
5510	*Klebsiella pneumoniae*	-	-	-
5511	*Klebsiella pneumoniae*	*bla*$_{OXA-181}$	-	+
5512	*Escherichia coli*	-	-	-
5513	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
5514	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-

5516	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
5517	*Escherichia coli*	-	-	-
5518	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	-	-
5519	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	-	-
5520	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
5521	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
5522	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
5524	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
5525	*Klebsiella pneumoniae*	*bla*$_{OXA-181}$	-	+
5526	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
5527	*Salmonella enteritidis*	-	-	-
5528	*Klebsiella pneumoniae*	-	-	-
4845	*Klebsiella pneumoniae*	-	-	-
4852	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
4855	*Klebsiella pneumoniae*	-	-	-
4856	*Klebsiella pneumoniae*	-	-	-
4857	*Klebsiella pneumoniae*	-	-	-
4859	*Klebsiella pneumoniae*	-	-	-
4860	*Klebsiella pneumoniae*	-	-	-
4861	*Klebsiella pneumoniae*	-	-	-
4862	*Klebsiella pneumoniae*	-	-	-
4864	*Klebsiella pneumoniae*	-	-	-
4865	*Klebsiella pneumoniae*	-	-	-
4867	*Klebsiella pneumoniae*	-	-	-
4869	*Klebsiella pneumoniae*	-	-	-
4871	*Klebsiella pneumoniae*	-	-	-
4874	*Klebsiella pneumoniae*	-	-	-
4878	*Klebsiella pneumoniae*	-	-	-
4879	*Klebsiella pneumoniae*	-	-	-
4880	*Klebsiella pneumoniae*	-	-	-
4882	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
4884	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
4885	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
4886	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
4887	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
4958	*Klebsiella pneumoniae*	-	-	-
3760	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	+	-
1000	*Klebsiella pneumoniae*	-	-	-
1001	*Klebsiella pneumoniae*	-	-	-

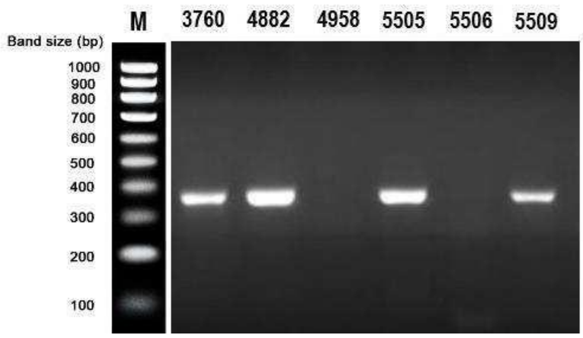

Figure 3.12- Representative image of the simplex PCR products for the *bla*$_{KPC}$ genes amplification. Agarose gel image show the amplification results of 6 strains (3760, 4882, 4958, 5505, 5506, 5509), listed above each well. The ladder used was the NZYDNA Ladder V, with the band sized of the DNA showed on the left. The expected size of the PCR products was 352bp for the *bla*$_{KPC}$ genes.

To test the multiplex PCR detection method of both genes' amplification, we applied the second set of primers. The results obtained revealed an efficient amplification of both genes (*bla*$_{KPC-3}$ and *bla*$_{OXA-48-like}$), in all 24 *K. pneumoniae* clinical isolates **(Figure 3.13)**.

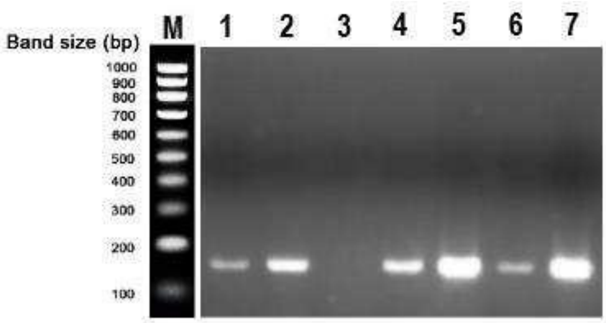

Figure 3.13- Representative image of the multiplex PCR products. Agarose gel image show the amplification results of 7 strains, listed above each well. The ladder used was the NZYDNA Ladder V, with the band sized of the DNA showed on the left. The expected size of the PCR products was 149bp for *bla*$_{KPC}$ and 150bp for *bla*$_{OXA}$ (*bla*$_{KPC}$ and *bla*$_{OXA}$). **Legend: 1-** 3760; **2-** 4882; **3-** 4958; **4-** 5505; **5-** 5506; **6-** 5509; **7-** 5511.

3.3. Ceftazidime-avibactam (CZA) antimicrobial susceptibility test in *Klebsiella pneumoniae* isolates

Given the recent emergence of *bla*$_{OXA-181}$ we further tested the resistance level to ceftazidime (CAZ) and CAZ-avibactam (CZA) given the more recent introduction of this drug-inhibitor combination in the treatment of carbapenemase-producing strains and to investigate if *bla*$_{OXA-181}$ was associated with increased resistance levels to this drug-inhibitor combination. The MIC of CAZ and CZA was therefore determined for five representative *K. pneumoniae* isolates, two *bla*$_{OXA-181}$ producers, two *bla*$_{KPC-3}$ producers, and one *bla*$_{KPC-3}$ and *bla*$_{GES-5}$ producer (**Table 3.10**).

Table 3.10- Description of all 5 *K. pneumonia* clinical isolates tested for CZA susceptibility and their MIC values. **Abbreviations:** MIC- minimum inhibitory concentration.

Strain ID	Microorganism	Carbapenemase coding gene known	MIC (mg/L)	
			CAZ	CZA
5506	*Klebsiella pneumoniae*	*bla*$_{OXA-181}$	>64	≤ 1
5511	*Klebsiella pneumoniae*	*bla*$_{OXA-181}$	>64	≤ 1
4887	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$, *bla*$_{GES-5}$	>64	2
3734	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	64	≤ 1
3826	*Klebsiella pneumoniae*	*bla*$_{KPC-3}$	64	2

No significant difference was observed regarding the resistance levels to CZA by isolates producing different carbapenemases, all of which exhibited MIC levels within the susceptibility range (≤8 mg/L) according to the current EUCAST breakpoints for CZA. Also, it is evident the effectiveness of the CZA combination, compared to the CAZ alone, for which all isolates showed resistance (MIC ≥ 64 mg/L).

A study from 2017, tested four isolates of *K. pneumoniae* producing OXA-48-like carbapenemase, showing MIC values of 1 mg/L to isolates positive for OXA-48 and OXA-181 (166), similar to ours with OXA-181 (MIC ≤ 1 mg/L). Many other articles state similar MIC values associated with *bla*$_{OXA-48-like}$ genes (166–169), all revealing susceptibility for CZA, suggesting that this CZA antibiotic combination is still effective against these genes. However, a recent article uncovers a possible resistance to CZA from OXA-48-like *K. pneumoniae* isolates, through mutations in proteins involved in efflux (AcrB/AcrD/EmrA/Mdt) and/or membrane permeability (OmpK36) without concomitant fitness cost mandating astute monitoring of ceftazidime-avibactam resistance among OXA-48 genotypes (170). Likewise, another study regarding the KPC-producing *K. pneumoniae* strain BO743 had as result a resistant MIC value of ≥ 256 mg/L for CZA. Interestingly, in this strain, several antimicrobial resistance genes were harbored, including *bla*$_{OXA-181}$ and *bla*$_{KPC-121}$, that were found in the pBO743-120Kbp and pBO743-54Kb plasmids, respectively. Also, the plasmid pBO743-54Kb showed high nucleotide identity to plasmids carrying the *bla*$_{OXA-181}$ gene isolated in several countries, as the pLB_OXA-181_PT109 (accession no. NZ_CP041033) isolated in Portugal and the pC872_2[NZ_CP067461 isolated in the United States (171). Therefore, more research should be done on the pLB OXA-181 PT109 plasmid and its impact on CZA resistance in Portugal.

Nevertheless, according to previous reports, it seems to exist also an association between increased levels of CZA resistance and the *bla*$_{KPC-3}$ genes (172–174). Most of these studies associated the CZA resistance with the KPC Ω loop that it is clearly a hot spot for mutations, modifying the β-lactamase hydrolytic properties, and thus putatively conferring CZA resistance. Warningly, some CZA-resistant KPC mutants were reported to capably revert to wild type upon exposure to carbapenems (172,173), associated with amino acid changes, specifically the D179Y (532G>T) in *K. pneumoniae*. This capacity highlights the flexible nature of such mutations, being even harder to overcome the CZA-resistant isolates carrying *bla*$_{KPC-3}$ genes, compared to the ones carrying *bla*$_{KPC-48-like}$. One strategy to overcome this problem described by (173), is the use of CZA combined with imipenem which resulted in the prevention of bacterial activity and the development of resistance against CZA.

4. Conclusion

This master thesis carried out a study regarding the global diversity and phylogenetic analyses, as well as the molecular detection of *bla*$_{KPC}$ and *bla*$_{OXA}$ genes. For the phylogenetic diversity analyses, we obtained 88 *bla*$_{KPC}$ and 428 *bla*$_{OXA}$ sequences from the available NCBI data, filtered by their carbapenemase activity. These sequences were later used in a programmatic tblastn for each allele against the entire nucleotide database at NCBI, for taxonomical dispersion analyses, obtaining a total of 1232 *bla*$_{KPC}$ and 2204 *bla*$_{OXA}$ hit accessions.

The diversity analyses, of the selected sequences, allowed the identification of, three KPC groups (KPC-2-like, KPC-3-like, and KPC-5-like) and five OXA groups (OXA-23-like, OXA-24/40-like, OXA-48-like, OXA-51-like, and OXA-58-like). It was also possible to observe that the KPC-2-like group encompasses the higher number of alleles, while among the OXA groups, the OXA-51-like was the one presenting a superior number of alleles. On the other hand, the taxonomical dispersion analyses across the 1232 *bla*$_{KPC}$ alleles exhibited 17 different genera associated with these genes with *Klebsiella*, *Escherichia,* and *Citrobacter* as the most common ones. KPC-2 and KPC-3 were the most disseminated coding genes, followed by KPC-4, KPC-5, and KPC-6 in third place. Regarding the taxonomical dispersion analyses of the 2204 *bla*$_{OXA}$ alleles, they have identified 15 different genera associated with these genes, with *Acinetobacter* as the most common, followed by *Klebsiella* and *Escherichia*. The OXA-51-like and OXA-48-like were the most prevalent groups, with OXA-48-like being also the most disseminated group, mostly the OXA-48 and OXA-181 coding genes. Moreover, taxonomical dispersion analyses have also identified one *bla*$_{OXA-51}$ gene (OXA-51_MK847907) in *Salmonella*, being the first report of a *bla*$_{OXA-51}$ gene present in this genus. These analyses have also identified, five *bla*$_{OXA-58}$ genes in a *Proteus*, with a common ancestor between all five, previously reported in Poland (MK086028), Belgium (KX668205, MK533136), and Germany (KU871397, KU871396).

In addition, we have also characterized the genetic context of 450 *bla*$_{KPC}$ alleles, discovering three Tn*4401* isoforms (Tn*4401a* (44,4%), Tn*4401b* (49,7%), Tn*4401d* (5,8%)), of which one *bla*$_{KPC-31}$ gene (CP043048) found in a Tn*4401d*. *Klebsiella* (61%), *Escherichia* (10%), and *Citrobacter* (9%) were the three most reported genera harboring a Tn*4401* isoform.

The second part of the study encompassed the molecular detection of *bla*$_{KPC-3}$ and *bla*$_{OXA-48-like}$ genes, through simplex and multiplex PCR. This study was performed on a set of 52 *Enterobacterales* clinical isolates, where 20 isolates were positive to *bla*$_{KPC-3}$

and 4 isolates positive to *bla*$_{OXA-181}$. In addition, a multiplex PCR was performed in a set of 24 *K. pneumoniae* isolates known to carry *bla*$_{OXA-181}$ or *bla*$_{KPC-3}$ genes, all of which were successfully amplified. Lastly, we also carried out an antimicrobial susceptibility test for CZA, where 5 *K. pneumoniae* isolates were tested, 2 of which carrying *bla*$_{OXA-181}$ genes, 2 others carrying *bla*$_{KPC-3}$ genes, and 1 carrying *bla*$_{KPC-3}$ and *bla*$_{GES-5}$ genes. All revealed susceptibility to CZA, with MIC values between ≤ 1 mg/L and 2 mg/L.

Overall, we were able to a) establish previously unreported KPC groups, highlighting the horizontal gene transfer capacity of *bla*$_{KPC}$ and *bla*$_{OXA}$ genes, b) report, for the first time, the presence of a *bla*$_{KPC-31}$ gene (CP043048) in a Tn*4401d*, and c) identify, the presence of a *bla*$_{OXA-51}$ gene (MK847907) in *Salmonella*. Furthermore, the detection method of both simplex and multiplex were generally successful, and no differences in the CZA antimicrobial susceptibility test was observed between the isolates producing different carbapenemases.

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6. Supplementary material

Supplementary Table S 6.1- Description of all 450 *bla*$_{KPC}$ genes and correspondent Tn*4401* isoforms identified, as well as the percentage of coverage and identity of each KPC hit accession.

***bla*$_{KPC}$ genes (KPC_accession)**	**Tn*4401* isoform**	**%Coverage**	**%Identity**
KPC-29_MT809695	Tn4401a	100.00	100.00
KPC-2_AP023266	Tn4401a	100.00	100.00
KPC-2_AP024751	Tn4401a	100.00	100.00
KPC-2_AP024754	Tn4401a	100.00	100.00
KPC-2_AP024757	Tn4401a	100.00	100.00
KPC-2_AP024759	Tn4401a	100.00	100.00
KPC-2_AP024761	Tn4401a	100.00	100.00
KPC-2_AP024764	Tn4401a	100.00	100.00
KPC-2_AP024767	Tn4401a	100.00	100.00
KPC-2_AP024770	Tn4401a	100.00	100.00
KPC-2_AP024773	Tn4401a	100.00	100.00
KPC-2_AP024776	Tn4401a	100.00	100.00
KPC-2_AP024777	Tn4401a	100.00	100.00
KPC-2_AP024779	Tn4401a	100.00	100.00
KPC-2_AP024782	Tn4401a	100.00	100.00
KPC-2_AP024786	Tn4401a	100.00	100.00
KPC-2_AP024790	Tn4401a	100.00	100.00
KPC-2_AP024793	Tn4401a	100.00	100.00
KPC-2_AP024796	Tn4401a	100.00	100.00
KPC-2_CP008798	Tn4401a	100.00	100.00
KPC-2_CP008833	Tn4401a	100.00	100.00
KPC-2_CP009876	Tn4401a	100.00	100.00
KPC-2_CP010362	Tn4401a	100.00	100.00
KPC-2_CP010396	Tn4401a	100.00	100.00
KPC-2_CP010575	Tn4401a	100.00	100.00
KPC-2_CP011981	Tn4401a	100.00	100.00
KPC-2_CP011986	Tn4401a	100.00	100.00
KPC-2_CP011991	Tn4401a	100.00	100.00
KPC-2_CP011999	Tn4401a	100.00	100.00
KPC-2_CP012000	Tn4401a	100.00	100.00
KPC-2_CP015347	Tn4401a	100.00	100.00
KPC-2_CP015382	Tn4401a	100.00	100.00
KPC-2_CP015395	Tn4401a	100.00	100.00
KPC-2_CP018356	Tn4401a	100.00	100.00
KPC-2_CP018949	Tn4401a	100.00	100.00
KPC-2_CP018989	Tn4401a	100.00	100.00
KPC-2_CP019017	Tn4401a	100.00	100.00
KPC-2_CP020110	Tn4401a	100.00	100.00
KPC-2_CP020500	Tn4401a	100.00	100.00
KPC-2_CP022574	Tn4401a	100.00	100.00

KPC-2_CP022693	Tn4401a	100.00	100.00
KPC-2_CP022698	Tn4401a	100.00	100.00
KPC-2_CP022917	Tn4401a	100.00	100.00
KPC-2_CP022920	Tn4401a	100.00	100.00
KPC-2_CP022923	Tn4401a	100.00	100.00
KPC-2_CP022926	Tn4401a	100.00	100.00
KPC-2_CP023443	Tn4401a	100.00	100.00
KPC-2_CP023928	Tn4401a	100.00	100.00
KPC-2_CP025006	Tn4401a	100.00	100.00
KPC-2_CP025009	Tn4401a	100.00	100.00
KPC-2_CP025010	Tn4401a	100.00	100.00
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KPC-2_CP027056	Tn4401a	100.00	100.00
KPC-2_CP027150	Tn4401a	100.00	100.00
KPC-2_CP027158	Tn4401a	100.00	100.00
KPC-2_CP030342	Tn4401a	100.00	100.00
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KPC-2_CP040384	Tn4401a	100.00	100.00
KPC-2_CP048384	Tn4401a	100.00	100.00
KPC-2_CP049601	Tn4401a	100.00	100.00
KPC-2_CP050075	Tn4401a	100.00	100.00
KPC-2_CP050168	Tn4401a	100.00	100.00
KPC-2_CP052170	Tn4401a	100.00	100.00
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KPC-2_CP056026	Tn4401a	100.00	100.00
KPC-2_CP059315	Tn4401a	100.00	100.00
KPC-2_CP068609	Tn4401a	100.00	100.00
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KPC-2_CP068617	Tn4401a	100.00	100.00
KPC-2_CP069560	Tn4401a	100.00	100.00
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KPC-2_CP070571	Tn4401a	100.00	100.00
KPC-2_CP070575	Tn4401a	100.00	100.00
KPC-2_CP070576	Tn4401a	100.00	100.00
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KPC-2_CP070599	Tn4401a	100.00	100.00
KPC-2_CP080654	Tn4401a	100.00	100.00
KPC-2_CP080663	Tn4401a	100.00	100.00

KPC-2_CP080668	Tn4401a	100.00	100.00
KPC-2_CP080672	Tn4401a	100.00	100.00
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KPC-2_CP080699	Tn4401a	100.00	100.00
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KPC-2_CP080719	Tn4401a	100.00	100.00
KPC-2_CP085731	Tn4401a	100.00	100.00
KPC-2_CP086668	Tn4401a	100.00	100.00
KPC-2_CP086675	Tn4401a	100.00	100.00
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KPC-2_HG969995	Tn4401a	100.00	100.00
KPC-2_HQ589350	Tn4401a	100.00	100.00
KPC-2_JX104759	Tn4401a	100.00	100.00
KPC-2_JX283456	Tn4401a	100.00	100.00
KPC-2_JX430448	Tn4401a	100.00	100.00
KPC-2_JX461340	Tn4401a	100.00	100.00
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KPC-2_KF874499	Tn4401a	100.00	100.00
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KPC-2_KJ146689	Tn4401a	100.00	100.00
KPC-2_KR052098	Tn4401a	100.00	100.00
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KPC-2_KU665642	Tn4401a	100.00	100.00
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KPC-2_KY798507	Tn4401a	100.00	100.00
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KPC-2_LT009688	Tn4401a	100.00	100.00
KPC-2_LT009689	Tn4401a	100.00	100.00
KPC-2_LT216438	Tn4401a	100.00	100.00
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KPC-2_MF497782	Tn4401a	100.00	100.00
KPC-2_MG557994	Tn4401a	100.00	100.00
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KPC-31_MT809691	Tn4401a	100.00	100.00
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KPC-31_MZ606382	Tn4401a	100.00	100.00
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KPC-3_CP009773	Tn4401d	100.00	100.00
KPC-3_CP009776	Tn4401d	100.00	100.00
KPC-3_CP010363	Tn4401d	100.00	100.00
KPC-3_CP010881	Tn4401d	100.00	100.00
KPC-3_CP014669	Tn4401a	100.00	100.00
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KPC-3_CP015387	Tn4401d	100.00	100.00
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KPC-3_CP061387	Tn4401a	100.00	100.00
KPC-3_CP061401	Tn4401d	100.00	100.00
KPC-3_CP068621	Tn4401a	100.00	100.00
KPC-3_CP070560	Tn4401a	100.00	100.00
KPC-3_CP070580	Tn4401a	100.00	100.00
KPC-3_CP070587	Tn4401a	100.00	100.00
KPC-3_CP083703	Tn4401a	100.00	100.00
KPC-3_GU595196	Tn4401a	100.00	100.00
KPC-3_HG969997	Tn4401a	100.00	100.00

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KPC-3_JX442975	Tn4401a	100.00	100.00
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KPC-3_KF954760	Tn4401d	100.00	100.00
KPC-3_KJ721789	Tn4401d	100.00	100.00
KPC-3_KJ721790	Tn4401a	100.00	100.00
KPC-3_KP345882	Tn4401d	100.00	100.00
KPC-3_KT362706	Tn4401a	100.00	100.00
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KPC-3_KY659388	Tn4401a	100.00	100.00
KPC-3_KY930324	Tn4401d	100.00	100.00
KPC-3_KY930325	Tn4401d	100.00	100.00
KPC-3_LT838197	Tn4401d	100.00	100.00
KPC-3_MH594478	Tn4401a	100.00	100.00
KPC-3_MT560075	Tn4401a	100.00	100.00
KPC-3_MT560078	Tn4401a	100.00	100.00
KPC-3_MT571488	Tn4401d	100.00	100.00
KPC-3_MT809688	Tn4401a	100.00	100.00
KPC-3_MT809689	Tn4401a	100.00	100.00
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KPC-53_CP058327	Tn4401a	100.00	100.00
KPC-53_CP058330	Tn4401a	100.00	100.00
KPC-55_MT028409	Tn4401a	100.00	100.00
KPC-66_MT809696	Tn4401a	100.00	100.00
KPC-66_MT809699	Tn4401a	100.00	100.00
KPC-67_MT809697	Tn4401a	100.00	100.00
KPC-67_MW650890	Tn4401a	100.00	100.00
KPC-67_MW650891	Tn4401a	100.00	100.00
KPC-68_MT809698	Tn4401a	100.00	100.00
KPC-69_MT809700	Tn4401a	100.00	100.00
KPC-70_MT809701	Tn4401a	100.00	100.00
KPC-14_CP045022	*Tn4401b*	100.00	99.80
KPC-18_KT884517	*Tn4401b*	100.00	99.59
KPC-6_CP027606	*Tn4401b*	100.00	99.80
KPC-5_JX193302	*Tn4401b*	100.00	99.80
KPC-4_CP020119	*Tn4401b*	100.00	99.80

KPC-4_CP020527	*Tn4401b*	100.00	99.80
KPC-4_CP021899	*Tn4401b*	100.00	99.80
KPC-4_CP024910	*Tn4401b*	100.00	99.80
KPC-4_CP029248	*Tn4401b*	100.00	99.80
KPC-4_CP034755	*Tn4401b*	100.00	99.80
KPC-4_CP072971	*Tn4401b*	100.00	99.80
KPC-4_JQ837276	*Tn4401b*	100.00	99.80
KPC-4_JX193301	*Tn4401b*	100.00	99.80
KPC-4_KR091915	*Tn4401b*	100.00	99.80
KPC-4_KX868553	*Tn4401b*	100.00	99.80
KPC-4_MF351991	*Tn4401b*	100.00	99.80
KPC-4_CP012168	*Tn4401b*	100.00	99.80
KPC-4_CP018352	*Tn4401b*	100.00	99.80
KPC-4_CP018675	*Tn4401b*	100.00	99.80
KPC-4_CP018676	*Tn4401b*	100.00	99.80
KPC-40_MK862125	*Tn4401b*	100.00	99.80
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KPC-3_KY093014	*Tn4401b*	100.00	99.80
KPC-3_JX500680	*Tn4401b*	100.00	99.80
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KPC-3_KJ748372	*Tn4401b*	100.00	99.80
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KPC-3_KU295131	*Tn4401b*	100.00	99.80
KPC-3_KU295134	*Tn4401b*	100.00	99.80
KPC-3_KU295136	*Tn4401b*	100.00	99.80
KPC-3_GU386376	Tn4401a	100.00	99.49
KPC-3_FJ223605	*Tn4401b*	100.00	99.80
KPC-3_CP072946	*Tn4401b*	100.00	99.80
KPC-3_CP072951	*Tn4401b*	100.00	99.80
KPC-3_CP072953	*Tn4401b*	100.00	99.80
KPC-3_CP072962	*Tn4401b*	100.00	99.80
KPC-3_CP073007	*Tn4401b*	100.00	99.80
KPC-3_CP073009	*Tn4401b*	100.00	99.80
KPC-3_CP073044	*Tn4401b*	100.00	99.80
KPC-3_CP073050	*Tn4401b*	100.00	99.80
KPC-3_CP073055	*Tn4401b*	100.00	99.80
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KPC-3_CP069046	*Tn4401b*	100.00	99.80
KPC-3_CP069050	*Tn4401b*	100.00	99.80
KPC-3_CP061406	*Tn4401b*	100.00	98.77
KPC-3_CP061394	*Tn4401b*	100.00	98.77
KPC-3_CP061398	*Tn4401b*	100.00	98.77
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KPC-3_CP060655	*Tn4401b*	100.00	99.80
KPC-3_CP060660	*Tn4401b*	100.00	99.80

KPC-3_CP060663	*Tn4401b*	100.00	99.80
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KPC-3_CP030335	*Tn4401b*	100.00	99.80
KPC-3_CP025710	*Tn4401b*	100.00	99.80
KPC-3_CP025758	*Tn4401b*	100.00	99.80
KPC-3_CP023875	*Tn4401b*	100.00	99.80
KPC-3_CP023878	*Tn4401b*	100.00	99.80
KPC-3_CP023894	*Tn4401b*	100.00	99.80
KPC-3_CP021860	*Tn4401b*	100.00	99.80
KPC-3_CP021861	*Tn4401b*	100.00	99.80
KPC-3_CP020059	*Tn4401b*	100.00	99.80
KPC-3_CP020066	*Tn4401b*	100.00	99.80
KPC-3_CP020075	*Tn4401b*	100.00	99.80
KPC-3_CP020359	*Tn4401b*	100.00	99.80
KPC-3_CP020503	*Tn4401b*	100.00	99.80
KPC-3_CP020507	*Tn4401b*	100.00	99.80
KPC-3_CP021546	*Tn4401b*	100.00	99.80
KPC-3_CP021548	*Tn4401b*	100.00	99.80
KPC-3_CP021716	*Tn4401b*	100.00	98.77
KPC-3_CP021734	*Tn4401b*	100.00	99.80
KPC-3_CP021754	*Tn4401b*	100.00	99.80
KPC-3_CP021756	*Tn4401b*	100.00	99.80
KPC-3_CP019010	*Tn4401b*	100.00	98.77
KPC-3_CP011575	*Tn4401b*	100.00	99.80
KPC-3_CP011578	*Tn4401b*	100.00	99.80
KPC-3_CP011589	*Tn4401b*	100.00	99.80
KPC-3_CP011645	*Tn4401b*	100.00	99.80
KPC-3_CP011646	*Tn4401b*	100.00	99.80
KPC-3_CP011978	*Tn4401b*	100.00	99.80
KPC-3_CP012163	*Tn4401b*	100.00	99.80
KPC-3_AM774409	*Tn4401b*	100.00	99.80
KPC-3_CP006919	*Tn4401b*	100.00	99.80
KPC-3_CP009864	*Tn4401b*	100.00	99.80
KPC-3_CP010387	*Tn4401b*	100.00	98.77
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KPC-2_MN336501	*Tn4401b*	100.00	99.80
KPC-2_MN082782	*Tn4401b*	100.00	99.80
KPC-2_KY128483	*Tn4401b*	100.00	99.80
KPC-2_KY128484	*Tn4401b*	100.00	99.80

KPC-2_MK412914	*Tn4401b*	100.00	99.80
KPC-2_MH514861	*Tn4401b*	100.00	99.80
KPC-2_MG886286	*Tn4401b*	100.00	99.80
KPC-2_MF327265	*Tn4401b*	100.00	99.80
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KPC-2_LC636066	*Tn4401b*	100.00	99.80
KPC-2_KX783439	*Tn4401b*	100.00	99.80
KPC-2_KX783441	*Tn4401b*	100.00	99.80
KPC-2_KY128483	*Tn4401b*	100.00	99.80
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KPC-2_CP017993	*Tn4401b*	100.00	99.80
KPC-2_CP018362	*Tn4401b*	100.00	99.80
KPC-2_CP018426	*Tn4401b*	100.00	99.80
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KPC-2_CP016381	*Tn4401b*	100.00	99.80
KPC-2_CP013325	*Tn4401b*	100.00	99.80
KPC-2_CP011656	*Tn4401b*	100.00	99.80
KPC-2_CP011608	*Tn4401b*	100.00	99.80
KPC-2_CP011611	*Tn4401b*	100.00	99.80
KPC-2_CP011622	*Tn4401b*	100.00	99.80
KPC-2_CP011635	*Tn4401b*	100.00	99.80
KPC-2_CP011639	*Tn4401b*	100.00	99.80
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KPC-2_CP008901	*Tn4401b*	100.00	99.80
KPC-2_CP009465	*Tn4401b*	100.00	99.80
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KPC-2_CP009862	*Tn4401b*	100.00	99.80
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KPC-2_CP007558	*Tn4401b*	100.00	99.80
KPC-2_CP007732	*Tn4401b*	100.00	99.80
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KPC-2_CP004366	*Tn4401b*	100.00	99.80
KPC-2_CP004367	*Tn4401b*	100.00	99.80
KPC-2_AF481906	*Tn4401b*	100.00	99.80
KPC-2_CP021743	*Tn4401b*	100.00	99.80
KPC-2_CP021750	*Tn4401b*	100.00	99.80
KPC-2_CP021853	*Tn4401b*	100.00	99.80
KPC-2_CP026123	*Tn4401b*	100.00	99.80
KPC-2_CP018977	*Tn4401b*	100.00	99.80
KPC-2_CP018963	*Tn4401b*	100.00	99.80
KPC-2_CP018668	*Tn4401b*	100.00	99.80
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KPC-2_CP018884	*Tn4401b*	100.00	99.80
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KPC-2_CP026169	*Tn4401b*	100.00	99.80
KPC-2_CP026175	*Tn4401b*	100.00	99.80
KPC-2_CP026179	*Tn4401b*	100.00	99.80
KPC-2_CP026188	*Tn4401b*	100.00	99.80
KPC-2_CP026193	*Tn4401b*	100.00	99.80
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KPC-2_CP026204	*Tn4401b*	100.00	99.80
KPC-2_CP026205	*Tn4401b*	100.00	99.80
KPC-2_CP026210	*Tn4401b*	100.00	99.80
KPC-2_CP026213	*Tn4401b*	100.00	99.80
KPC-2_CP026216	*Tn4401b*	100.00	99.80
KPC-2_CP026225	*Tn4401b*	100.00	99.80
KPC-2_CP026227	*Tn4401b*	100.00	99.80
KPC-2_CP026230	*Tn4401b*	100.00	99.80
KPC-2_CP026232	*Tn4401b*	100.00	99.80
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KPC-2_CP026401	*Tn4401b*	100.00	99.80
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KPC-2_CP029446	*Tn4401b*	100.00	99.80
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KPC-2_CP037737	*Tn4401b*	100.00	99.80
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KPC-2_CP081316	*Tn4401b*	100.00	99.80
KPC-2_CP081352	*Tn4401b*	100.00	99.80
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KPC-2_EU176012	*Tn4401b*	100.00	99.80
KPC-2_EU176013	*Tn4401b*	100.00	99.80
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KPC-2_FJ223607	*Tn4401b*	100.00	99.80
KPC-2_JX397875	*Tn4401b*	100.00	99.80
KPC-2_JX500679	*Tn4401b*	100.00	99.80
KPC-2_KC609323	*Tn4401b*	100.00	99.80
KPC-2_KJ933392	*Tn4401b*	100.00	99.80
KPC-2_KM983022	*Tn4401b*	100.00	99.80
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KPC-2_KX397572	*Tn4401b*	100.00	99.80
KPC-2_KU295135	*Tn4401b*	100.00	99.80