The role of mobile genetic elements in the dissemination of resistance genes in *Klebsiella* spp. clinical strains

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Thesis to obtain the Master of Science Degree in

Microbiology

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November 2023

I declare that this document is an original work of my own authorship and that it fulfils all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa

Preface

The work presented in this thesis was performed at the EnviHealthMicro Lab, Institute of Environmental Health, University of Lisbon (Lisbon, Portugal), during the period September 2022 - October 2023, under the supervision of Prof. Dr. Cátia Caneiras. The thesis was co-supervised at Instituto Superior Técnico by Prof. Dr. Leonilde Moreira.

Scientific outcomes resulting from the masters' dissertation

1. **Ramalho, J. F.**; Mendes, G; Santos, M. L.; Duarte, A.; Caneiras, C. The role of mobile genetic elements in carbapenemase-producing *Klebsiella* spp. *Frontiers in Microbiology* (under revision)

Acknowledgments

First of all, I would like to thank my supervisor, Prof. Dr. Cátia Caneiras, for introducing me to the enormous world that is antibiotic resistance research. I am truly grateful for always being there to encourage me, support my ideas, and provide the best guidance for the development of this project.

I also want to express my heartfelt gratitude to all my laboratory colleagues: Gabriel Mendes, who went beyond helping me by teaching me all the laboratory techniques I needed for this project, for always being there for support, making long days look shorter, and exchange of ideas every time I needed; and Leonor Santos, who was always ready to help me even before I asked, to be an amazing example of self-sacrifice, selflessness, and that nothing is impossible.

I feel very lucky to have my family always providing me with the means to be successful. Thank you for your unconditional support. In particular, my mother, who taught me to smile even when it seems to be no reason for it, my father, for being the greatest example in my life, and my aunt, for being my second mother and whom I feel confident to tell news first-hand. A special thanks goes to Raquel Gouveia, her presence is enough to fulfill my day.

And because life without friends is like a sky without stars, I also want to thank them. My partners in crime: Eduardo Medeiros, Francisco Albardeiro, João Braguez, and João Madeira. I thank my far-away friends, Roberto Bullitta, and Leonardo Durão. Thank you to the game-night gang, Énia Nóbrega, Filipe Jorge, and Márcia Oliveira,

A final thanks to my athletics team, in particular my coach Ermelinda Mineiro, because sometimes all I need is a run.

Abstract

Healthcare-associated infections (HAIs) represent a significant health threat. *Klebsiella* species are among the leading causes, with carbapenem resistance becoming increasingly prevalent. This study includes 1140 strains from three hospital centers in Portugal. *Klebsiella pneumoniae* was identified as the predominant species (93.3%). This study also reveals the presence of other members of the *K. pneumoniae* species complex (*Kp*SC) (n=8), *Klebsiella aerogenes* (n=17), and *Klebsiella oxytoca* species complex (*Ko*SC) (n=10). A strong correlation was found between lineages and the main carbapenemases produced. The *bla*$_{OXA-181}$ gene was associated with *K. pneumoniae* ST17-KL25-O5 and ST147-KL64-O2 strains, and in *K. aerogenes* ST93-O2 strains. And the *bla*$_{KPC-3}$ gene in ST147-KL64-O2 and ST13-KL3-O1 *K. pneumoniae*. All ST2-KL74 *K. oxytoca* strains encoded *bla*$_{OXA-48}$ and co-production of two distinct carbapenemases was confirmed in all three species groups. Despite this, IncF and Col-like were the most represented plasmid families in all *Klebsiella* species, and different strains shared similar *bla*$_{KPC}$- and bla$_{OXA-181}$-harbouring plasmids. Moreover, these genes were found within the Tn*4401*d and IS26-like transposon, respectively. The study also reports a novel carbapenemase, KPC-98, which confers resistance to ceftazidime-avibactam, and is related with a high fitness cost, leading to the loss of the resistance gene over time, highlighting the dynamic nature of resistance mechanisms. This research offers insights into the complex landscape of carbapenem resistance in *Klebsiella* species in Portuguese healthcare facilities, emphasizing the urgent need for continuous surveillance of antimicrobial resistance, as well as the importance of understanding the role of mobile genetic elements (MGE) in resistance dissemination.

Keywords: carbapenem resistance; *Klebsiella* genus; carbapenemases; mobile genetic elements; genomic surveillance

Resumo

As infeções associadas aos cuidados de saúde (IACS) representam uma ameaça à saúde. As espécies de *Klebsiella* resistentes aos carbapenemos estão entre as principais causas. Este estudo inclui 1140 estirpes provenientes de três centros hospitalares em Portugal. *Klebsiella pneumoniae* foi identificada como espécie predominante (93,3%). Este estudo também revela a presença de outros membros do complexo de espécies *K. pneumoniae* (*Kp*SC) (n=8), *Klebsiella aerogenes* (n=17) e complexo de espécies *Klebsiella oxytoca* (*Ko*SC) (n=10). Foi encontrada forte correlação entre linhagens e as principais carbapenemases produzidas. O gene *bla*$_{\text{OXA-181}}$ foi associado às linhagens ST17-KL25-O5 e ST147-KL64-O2 de *K. pneumoniae* e à linhagem ST93-O2 de *K. aerogenes*. E o gene *bla*$_{\text{KPC-3}}$ nas linhagens ST147-KL64-O2 e ST13-KL3-O1 de *K. pneumoniae*. Todas as estirpes ST2-KL74 *K. oxytoca* codificaram *bla*$_{\text{OXA-48}}$ e a coprodução de duas carbapenemases distintas foi confirmada em todos os grupos de espécies. Apesar disso, IncF e Col-like foram as famílias de plasmídeos mais representadas, e diferentes estirpes compartilharam plasmídeos semelhantes contendo *bla*$_{\text{KPC}}$ e *bla*$_{\text{OXA-181}}$. Além disso, esses genes foram encontrados no transposão Tn*4401*d e IS26, respetivamente. O estudo também relata uma nova carbapenemase, KPC-98, que confere resistência à ceftazidima-avibactam e está relacionada com um alto custo de fitness, levando à perda do gene de resistência ao longo do tempo. Este trabalho oferece informações sobre o panorama da resistência aos carbapenemos em espécies de *Klebsiella* em hospitais portugueses, destacando a necessidade urgente de vigilância à resistência antimicrobiana, bem como a importância de compreender o papel dos elementos genéticos móveis (MGE) na disseminação da resistência.

Palavras-chave: resistência aos carbapenemos; género *Klebsiella*; carbapenemases; elementos genéticos móveis; vigilância genómica

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List of Abbreviations

ADE - Distilled water
AMR - Antimicrobial resistance
AST - Antimicrobial Susceptibility Testing
BHI - Brain Heart Infusion
BLAST - Basic Local Alignment Search Tool
BRIG - BLAST Ring Image Generator
CPE - Carbapenemase-Producing Enterobacterales
CDC - Centers for Disease Control and Prevention
CG - Clonal group
CZA - Ceftazidime-avibactam
CTX-M - Cefotaximase-Munich β-lactamase
DNA - Deoxyribonucleic acid
ECDC - European Centre for Disease Prevention and Control
ESBL - Extended-Spectrum Beta-Lactamase
EU - European Union
EUCAST - European Committee on Antimicrobial Susceptibility Testing
GES - Guiana extended-spectrum β-lactamase
HAIs - Healthcare-associated infections (HAIs)
HGT - Horizontal Gene Transfer
ICE - Integrative and conjugative element
IMP - Imipenemase
intl - Integrons
IR - Inverted repeats
ISAba125 - Insertion sequence of *Acinetobacter*
K-locus - Capsular locus
Ka - *Klebsiella* aerogenes
KPC - *Klebsiella pneumoniae* carbapenemase
KpSC - *Klebsiella pneumoniae* species complex
KoSC - *Klebsiella oxytoca* species complex
LB - Luria-Bertani
MDR - Multi-drug resistance
MEM - Meropenem
MIC - Minimum Inhibitory Concentration
MLST - Multi-locus sequence typing
MRSA - Methicillin-Resistant *Staphylococcus aureus*
MSSA - Methicillin-Susceptible *Staphylococcus aureus*
NDM - New Dehli metallo-β-lactamase
NTE_{KPC} - Non Tn*4401* element

OXA - Oxacilinase-48
O-locus - O-antigen locus
PacBio - Pacific Biosciences
PCR - Polymerase Chain Reaction
qnr - Quinolone resistance gene
RNA - Ribonucleic Acid
spp. - Species (plural)
ST - Sequence type
TBE - Tris-Borate-EDTA buffer
TEM - Temoneira β-lactamase
Tn - Transposon
ULisboa - University of Lisbon
VIM - Verona integron-encoded metallo-β-lactamase
WHO - World Health Organization
WGS - Whole Genome Sequencing
gDNA - Genomic DNA
IPM - Imipenem
kb - Kilo basepairs

1. Introduction

1.1. *Klebsiella* genus: a lethal Healthcare-associated infection agent

Healthcare-associated infections (HAIs) are a significant concern in modern healthcare. These infections, acquired during medical treatment or while receiving care in a healthcare facility, can lead to prolonged hospital stays, increased healthcare costs, and, most importantly, patient morbidity and mortality [1]. A total of 8.9 million HAIs were estimated to occur each year in European hospitals and long-term care facilities combined [2].

Bacteria belonging to the *Klebsiella* genus are one of the main agents of these infections, including respiratory tract, urinary tract, and bloodstream infections. These Gram-negative bacteria belong to the *Enterobacteriaceae* family, are rod-shaped with a polysaccharide capsule, non-motile, facultative anaerobes and may behave as opportunistic human pathogens, mainly leading to pneumonia, sepsis, and urinary tract infections [3]. Among its species, *Klebsiella pneumoniae* is the most commonly identified in human infections, although other species, such as *Klebsiella quasipneumoniae*, *Klebsiella variicola*, *Klebsiella oxytoca*, *Klebsiella michiganensis*, *Klebsiella pasteurii*, *Klebsiella grimontii*, and *Klebsiella aerogenes*, formerly known as *Enterobacter aerogenes* [4] (**Figure 1**), have also been linked to nosocomial infections [5]. Moreover, *Klebsiella*-associated infections are strongly linked with hospitalization and high mortality rates through several nosocomial outbreaks in clinical settings [6]. Despite the improvements in recent years, Portugal has been identified as one of the countries in Europe with the highest rate of hospital infections [7], and with an increasing resistance tendency to the latest therapeutic lines available [8].

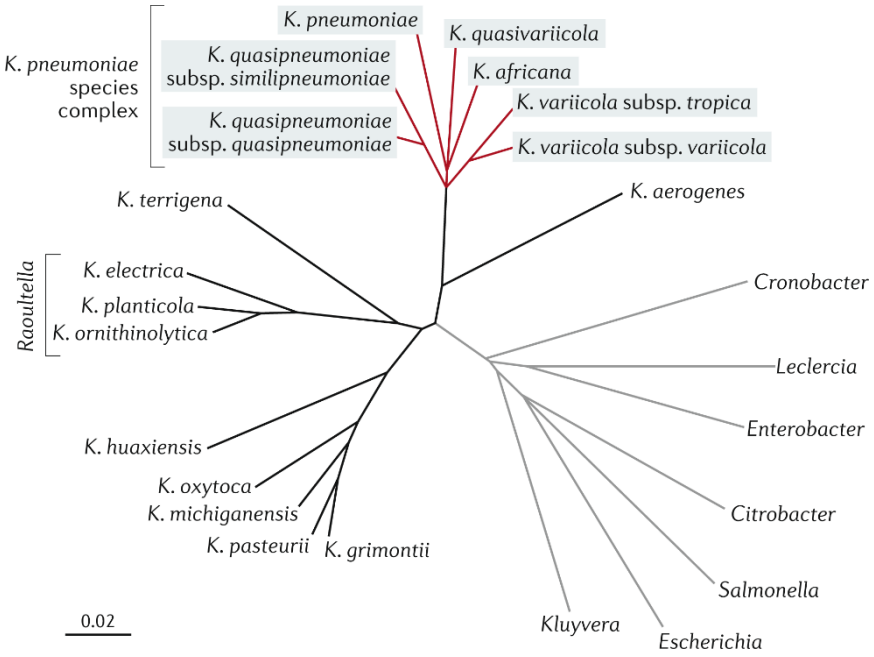

Figure 1 - Phylogenetic tree showing the relationships between *K. pneumoniae*, its close relatives in the *K. pneumoniae* species complex (red branches), other members of the *Klebsiella* genus (black branches) and family Enterobacteriaceae (grey branches).

1.2. The emergence of antibiotic-resistant strains

There has been a recent emergence of drug-resistant pathogenic strains, with over 33,000 deaths occurring each year from an infection caused by antibiotic-resistant bacteria [7]. The most common antibiotics prescribed for difficult-to-treat *Klebsiella spp.* infections are carbapenems, which act by inhibiting the cell wall biosynthesis in the bacterial organism [9]. The World Health Organization (WHO) had previously defined carbapenem-resistant Enterobacterales as one of the most critical global health threats [10]. The decrease in the outer membrane permeability due to a deficient porin expression and/or overexpression of porins that have a very weak affinity for carbapenems, the presence of efflux pumps that move the antibiotic to the outside of the bacteria, and, most importantly, the acquisition of genes that encode carbapenemases with hydrolytic effect against carbapenems, are among the main mechanisms that provide resistance against carbapenems in *Klebsiella spp.* strains (**Figure 2**). Within the carbapenemase genes reported on Enterobacteriaceae clinical strains, *bla*$_{KPC}$, *bla*$_{NDM}$, and *bla*$_{OXA\text{-}48}$-like are the most frequently found ones, which are mainly associated with mobile genetic elements (MGEs) such as plasmids, transposons, and integrons [11]. Overall, members of the *Klebsiella* genus are notorious for their high frequency and diversity of antimicrobial resistance (AMR) genes, as well as their ability to transmit them to other clinically important gram-negative bacteria [12,13].

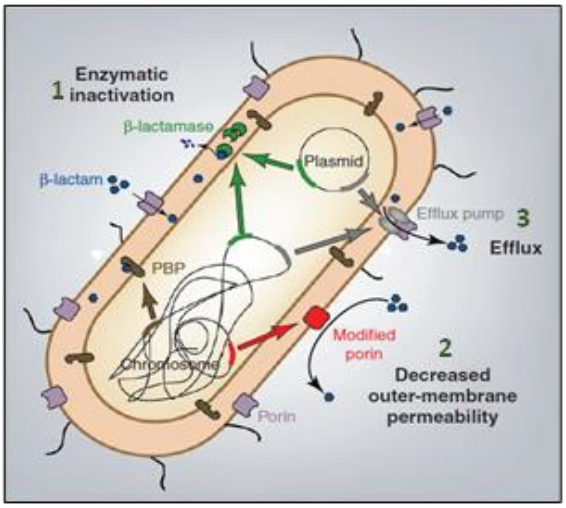

Figure 2 - Primary mechanisms of β-lactam resistance in Enterobacterales. Adapted from Nordmann et al, 2012.

1.3. A "One Health" approach

Recently, it has been made an increasing global effort on the revolutionary approach of "One Health" [14] – the intersection point between human, animal, and environmental health – in order to identify and prevent AMR spreading, since bacterial strains can move between niches in the environment, human and animal hosts, carrying with them virulence or resistance genes and/or plasmids (**Figure 3**) [12].

In the last years, Whole Genome Sequencing (WGS) technology have become faster and more accessible, allowing a better genomic surveillance of healthcare-associated infections by *K. pneumoniae*. Nevertheless, the interplay between resistance and MGEs in clinical strains remains poorly understood [15], and little is known on the role of these same MGE in the recent emergence of non-*K. pneumoniae* strains.

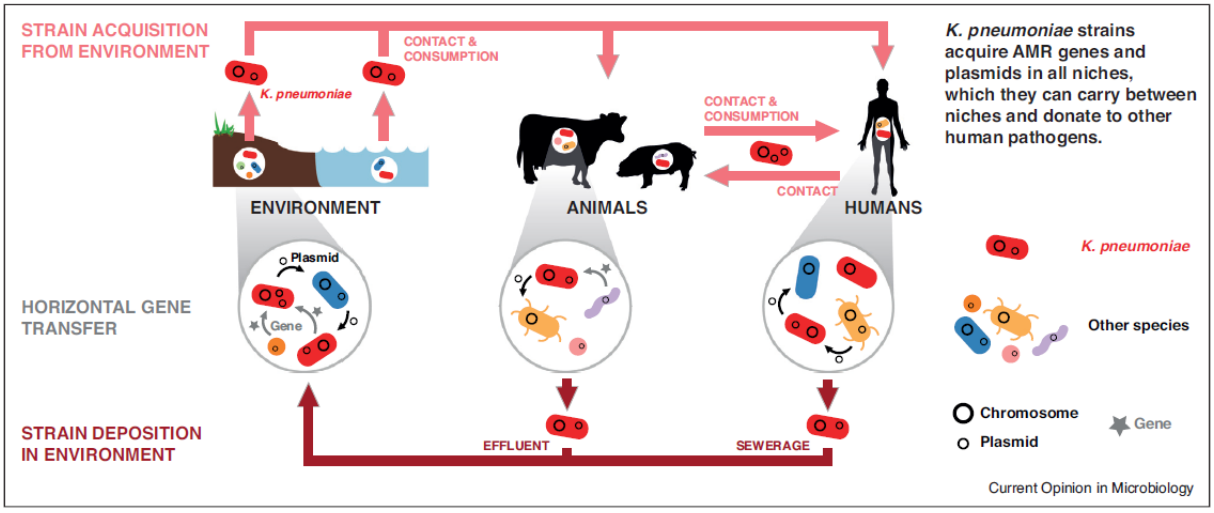

Figure 3 - AMR gene and plasmid trafficking by *K. pneumoniae*. Adapted from Wyres et al, 2018.

1.4. Mobile Genetic Elements in *Klebsiella* spp.

Klebsiella spp. have gained the capacity to acquire drug resistance phenotype mainly through horizontal gene transfer (HGT) of carbapenemases [11]. HGT is mediated by several well-described mechanisms [16] such as conjugation, the most significant one, but also transduction and transformation. The transfer of antibiotic resistance genes and their accumulation into multidrug-resistant clones has become a serious threat to human health thanks to bacterial mobile genetic elements (MGEs) such as plasmids, integrons, and transposons (**Figure 4**).

Bacterial conjugation requires physical contact between cells, it is unidirectional, and it is a genetically determined transfer mechanism, meaning that plasmid genes are required, which have no homologs on the chromosome. In conjugation, the donor cell produces a pilus that attaches to the bacterial recipient and brings both cells closer. The plasmid is then prepared for transfer, and it is nicked in a single strand. Next, a pore is formed between the two cells, and the plasmid strand moves from the donor to the recipient. At last, the single plasmid chains, both the one in the donor and the other in the recipient, re-circularize themselves and synthesize another chain, restoring the plasmid to its double-stranded constitution [17].

The transduction process involves bacteriophages in order to transfer chromosomal or plasmid genes from one bacterium to another. This mechanism of horizontal gene transfer can be divided into two main infection steps. In the first step, the donor bacteria are infected with virulent particles, which multiplicates and incorporates some host DNA inside the cell – lytic cycle, before being released to the outside. In the next stage, recipient cells are infected by these transducer particles and release donor DNA into the recipient which can be recombined into the chromosome if the bacterium contains the *recA* gene, resulting in a bacterial transductant [17].

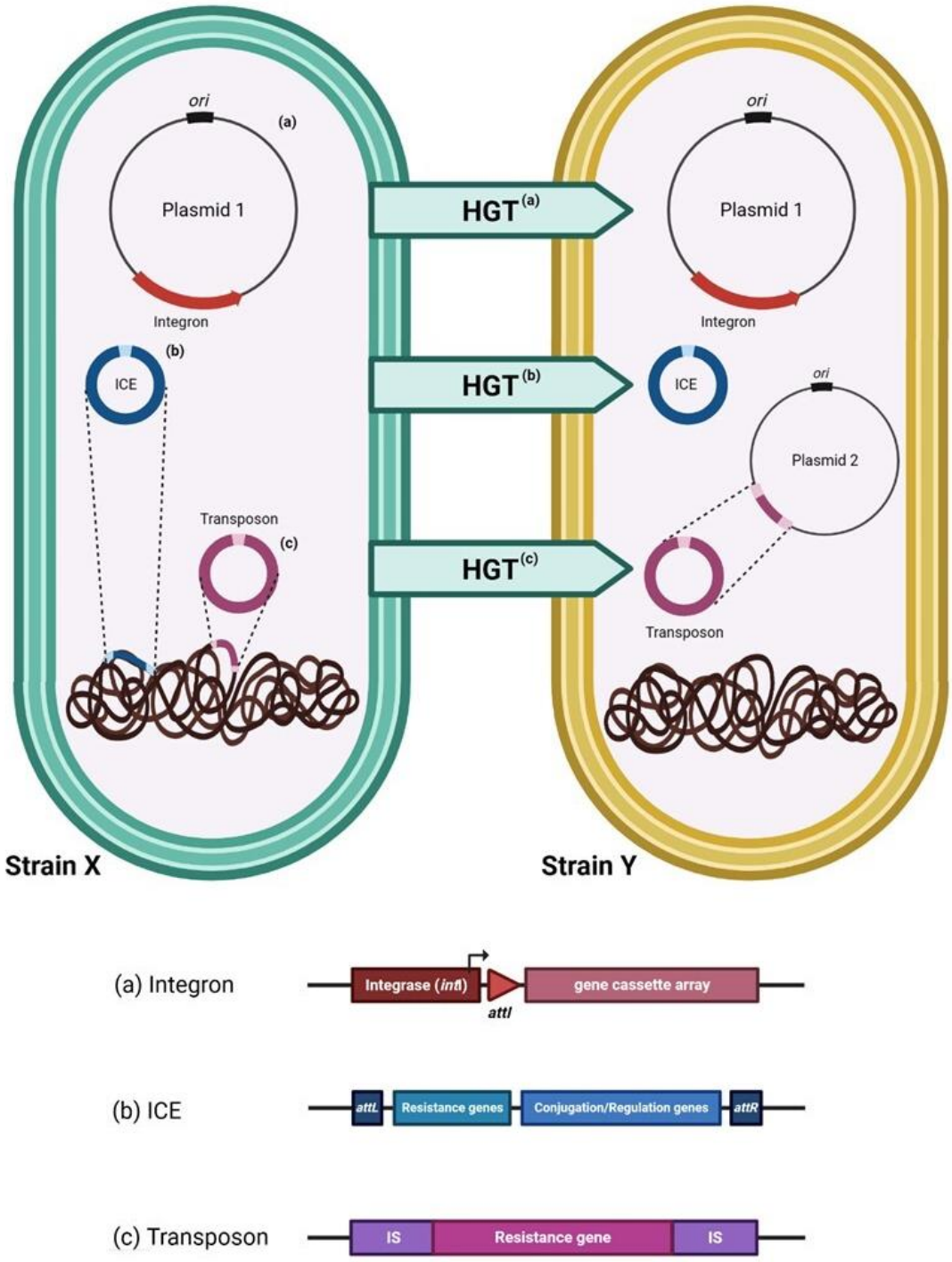

Figure 4 - Mobile genetic elements in *Klebsiella* spp. and their mechanisms horizontal gene transfer (HGT) of antibiotic resistance genes. (a) Integrons with resistance genes may move between plasmids and genomes of differente strains. (b) An integrative and conjugative element (ICE) can be integrated into the chromosome or excised and conjugate into another bacterium and integrate into the chromosome. (c) Insertion sequences (IS) flanking resistance genes allow it to move between the genome and plasmids of different cells as part of a composite transposon.

In transformation, released fragments of DNA from the donor cell are taken up directly from the extracellular environment by recipient bacteria. For transformation to occur, the recipient must first develop competence (temporary state), followed by adsorption, binding, and uptake of the genetic fragment mediated by very specialized proteins, then, a pre-integration complex is formed in the homology region and the donor DNA is recombined in the chromosome, with the assimilation of the exogenous chain and degradation of the other strand [18].

1.4.1. Integrons

An integron is a mobile structure that consists of a 5' conserved region - which contains the *int* gene for integrase, the recombination site, and a promoter that controls the transcription of the variable region -, the conserved 3' region, and a variable region which may contain antibiotic resistance genes, thus playing a significant role in antibiotic resistance dissemination, mainly among Gram-negative bacteria [19].

1.4.2. Insertion sequences

Insertion sequence elements (ISs) usually encode a transposase gene within inverted repeats at the 3' and 5. When it inserts in the genome, short direct repeats of the target DNA are created. This transposable element "jumps" from one location to another, it can leave a copy (replicative) or not (non-replicative) in the initial target site [20]. Previous studies have demonstrated an evident interaction between conjugative plasmids and ISs in the spread of antibiotic-resistance genes and, in some cases such as the IS6 family, interspecies HGT [21].

1.4.3. Transposons

Transposons are DNA sequences that contain a transposase flanked by ISs which also play an important for the migration of this element [22]. Associated with this kind of MGE, it is possible to find genetic cassettes and integrons. Transposons have the ability to move within the chromosome, within the plasmid, and between these two [23]. In a conjugative transposon, a closed circular intermediate is formed after excision, which can reintegrate into the bacterial genome or be transferred to other cells by conjugation [24].

1.4.4. Plasmids

Plasmids are small circular molecules of extrachromosomal DNA that can replicate autonomously due to their characteristic replicon – a small sequence that encodes the origin of replication (*ori*) and other replication initiation proteins. They have a variety of genes that may provide the bacterial additional advantage under certain environmental conditions at the cost of the cell fitness, including antibiotic resistance genes using other MGEs [25]. In 2005, a plasmid typing system was created based on the replicon sequence [26].

1.4.5. ICEs

Integrative and conjugative elements (ICEs) are self-transmissible MGEs, which usually contain a gene cassette that encodes virulence, antibiotic, and/or heavy metal resistance determinants [27]. The recombination mechanism of ICEs is similar to the one of conjugative plasmids, although, unlike plasmids, ICEs are stably maintained in the host genome. Integration may occur at a single site, or in several locations in the genome shared by the same class of ICEs [28].

1.5. Epidemiology of carbapenemase-encoding plasmids

To the best of our knowledge, there's no recent study that describes the epidemiology of carbapenemase-encoding plasmids among *Klebsiella spp*. Nevertheless, IncF, IncI, IncA/C, and IncH are some of the most common replicons described in Enterobacterales clinical strains [29], while IncA/C, IncL/M, IncF, IncI1, IncN were previously associated with carbapenemases [30].

In order to get a global perspective of global carbapenemase gene distribution within the different replicons, 148 complete sequenced plasmids were retrieved from the Genbank database, and the identification of resistance genes and replicon sequences was performed through ResFinder and PlasmidFinder, respectively (**Supplementary Table 1**). These tools are available at the "Center for Genomic Epidemiology" (https://www.genomicepidemiology.org/). The strains were mainly *K. pneumoniae* (138/148, 93%), followed by *K. quasipneumoniae* (5/148, 3%), *K. aerogenes*, (3/148, 2%), *K. michiganensis*, and *K. variicola* (1/148, 1%). Only *K. aerogenes* and *K. michiganensis* do not belong to the *K. pneumoniae* species complex [4]. These bacteria were mainly extracted from clinical context (144/148, 97%), being the rest from animal or environmental sources.

From this data, it is possible to detect a clear predominance of bla_{NDM}-like genes in Asia, bla_{KPC-3} and bla_{OXA-48} in Europe, and bla_{KPC-2} in America (**Figure 5A**). A large variety of resistance genes was found within the Asian continent. These results are consistent with recent studies on carbapenemase distribution among Enterobacterales clinical strains [31]. Despite this clear distinctive resistance profile between the different continents, the same cannot be observed for replicon distribution (**Figure 5B**) as IncFIB and IncR plasmids were the most frequently found across the globe.

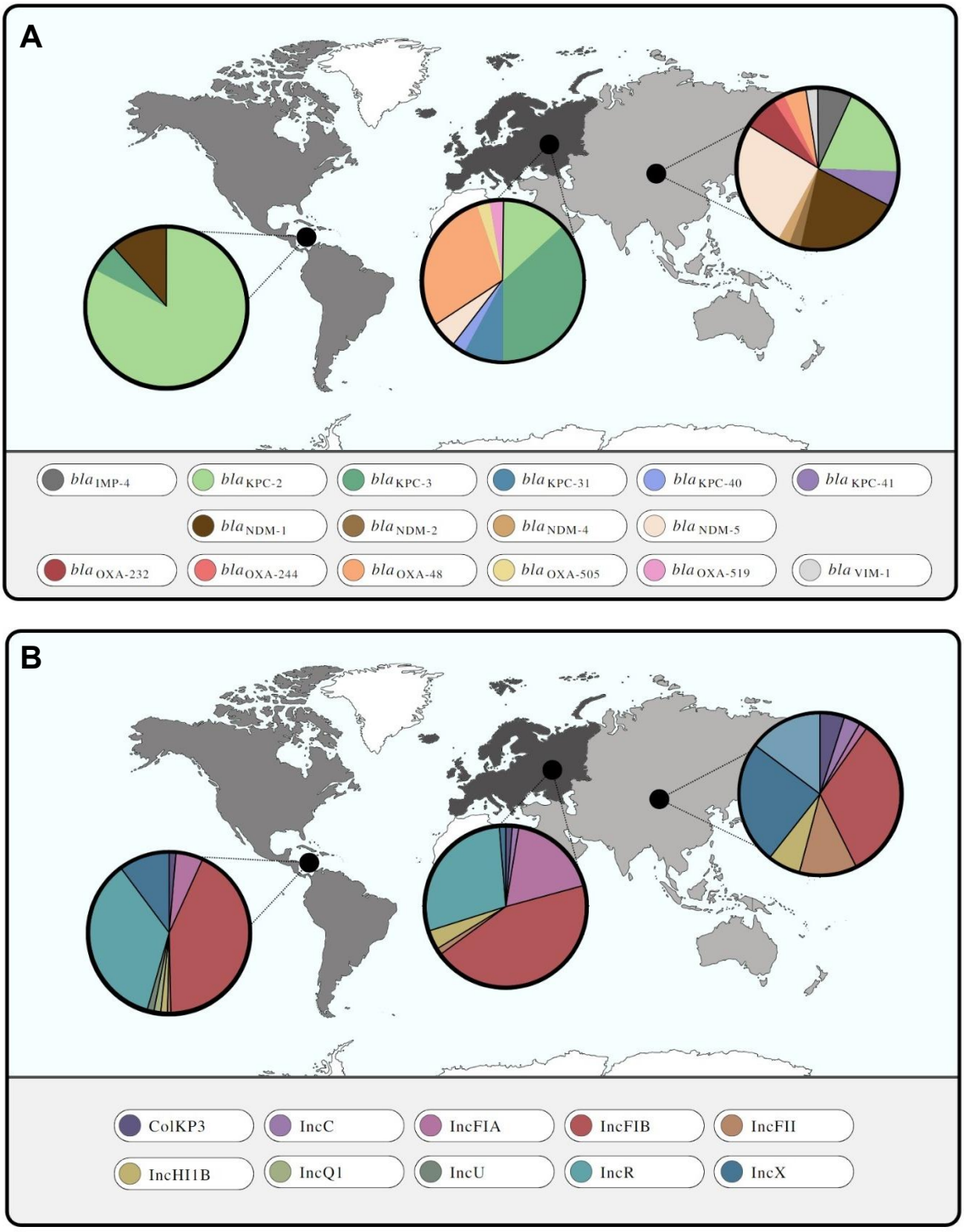

Figure 5 - Worldwide distribution of carbapenemase genes (**A**) encoded in different replicon types (**B**). Data retrieved from the Genbank database of 148 strains that were collected in the past 20 years. The African continent was excluded in this study due to a lack of a significant number of sequences.

Nevertheless, IncX was regularly described in American and Asian strains, whereas IncFIA was frequent in Europe. When trying to find a correlation between carbapenemase genes and replicons (**Figure 6**), it can be observed that the *bla*$_{KPC-2}$ is most common resistance gene across the main replicon types, while *bla*$_{KPC-3}$ is more strongly associated with the IncFIB incompatibility group. In fact, only plasmids containing, at least, the IncFIB replicon harboured the *bla*$_{KPC-3}$ gene. The *bla*$_{NDM-1}$

carbapenemase was also frequently found in IncFIB plasmids but also in many plasmids without any replicon type associated, whereas *bla*$_{NDM-5}$ was commonly found in IncFIB and IncX plasmids. IncX was also the replicon with less variety of associated carbapenemases.

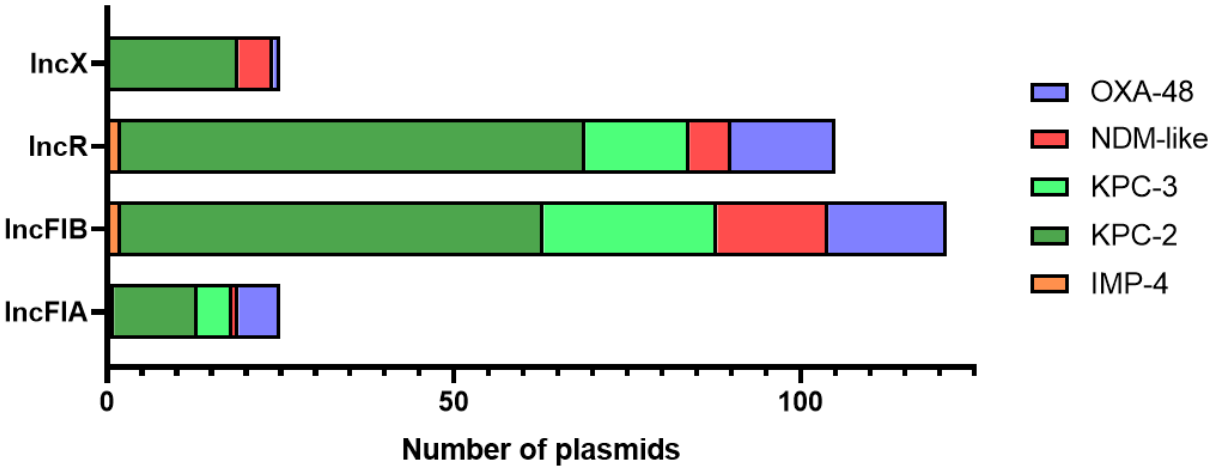

Figure 6 - Distribution of carbapenemase genes among the most frequently found replicon types.

1.6. Role of MGE on carbapenemase dissemination

1.6.1. KPC-like

The *bla*$_{KPC}$ gene is found primarily in Enterobacterales (majority in *K. pneumoniae*), belongs to Ambler Class A serine β-lactamase, hydrolyses all β-lactams (penicillins, extended-spectrum cephalosporins, aztreonam, and carbapenems) and it is inhibited by avibactam, vaborbactam and other novel β-lactamase inhibitors. The gene is typically contained on a plasmid within a transposon [32,33].

The pKpQIL plasmid was the first *bla*$_{KPC}$-bearing plasmid identified from *K. pneumoniae* ST258. The plasmid is over 100 kb in size, belongs to the IncFIIK/IncFIB(pQil) group, and contains the Tn*4401*a element [34]. Later, the pKp048 plasmid, an IncFIIK/IncR *bla*$_{KPC}$-harbouring plasmid, was found widely disseminated in China [35] and commonly associated with ST11, but this time, within a non-Tn*4401* element (NTE$_{KPC}$). Moreover, *bla*$_{KPC}$-bearing plasmids also frequently harbor quinolone (*qnrA*, *qnrB*), and aminoglycoside (*ArmA*, *RmtB*) resistance genes [36].

Tn*4401* is 10 kb in length, flanked by a 5-bp target-site duplication (TSD), and it harbors *bla*$_{KPC}$, a Tn3 transposase (*tnp*A), a Tn3 resolvase (*tnp*R), and two insertion sequences (ISKpn6, ISKpn7) flanked by two 39-bp inverted repeats (IR). So far, 9 isoforms of Tn*4401* (a-i) have been described in *Klebsiella spp.* strains, being Tn*4401*a and Tn*4401*b the most found ones. The main genetic variety occurs within a small region between ISKpn7 and the *bla*$_{KPC}$ gene (**Figure 7**). These mutations resulted in an increase in promoter activity, for instance, there's a 23-fold increase in KPC expression in Tn*4401*a, compared to the Tn*4401*b isoform [37]. Moreover, it was described a strong link between multiple copies of the Tn*4401* transposon and increased antibiotic resistance [38]. Lastly, this element can also be circulating in animal niches: in Greece, carbapenem-resistant *K. pneumoniae* ST709 strains containing pKpQIL-like plasmids with a *bla*$_{KPC-3}$-Tn*4401*a were identified in a veterinary hospital. These plasmids showed high similarity with the ones identified in high-risk clinical clones.

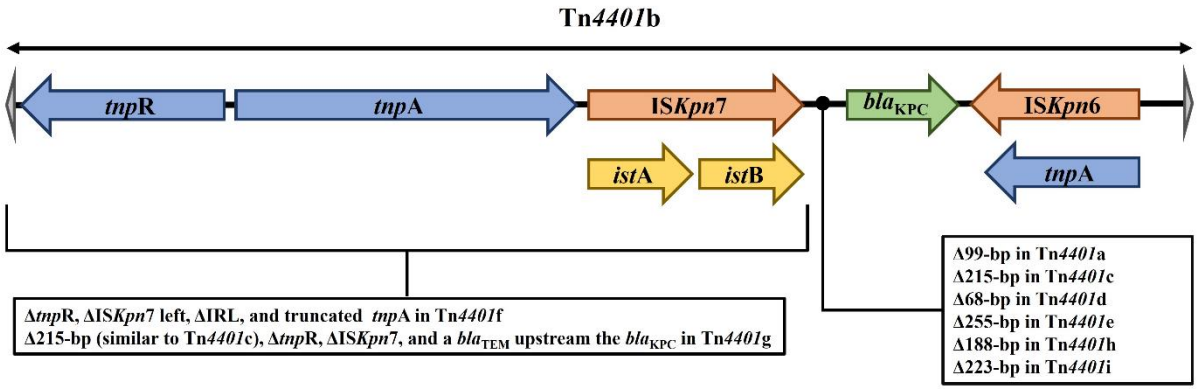

Figure 7 - Tn*4401* isoforms scheme.

The *bla*KPC has also been commonly described in other non-Tn*4401* elements (NTE$_{KPC}$), particularly in South America and Asia [39]. The ISKpn6-associated left IR (IRL) is intact (or partially) in NTE$_{KPC}$, which indicates a possible evolution from Tn*4401*. These elements are divided into two major groups, depending on the absence (NTE$_{KPC}$-I) or presence (NTE$_{KPC}$-II) of the *bla*$_{TEM}$ gene [40]. Within each group, several subgroups have been described, with distinct genetic backgrounds regarding different insertion sequences, the structure of the resolvase, and transposase genes, among other factors [41]. Remarkably, NTE$_{KPC}$s are commonly found in non-CG258 *K. pneumoniae* and/or other *Klebsiella* species in South America and Asia [39,42], while Tn*4401* is mostly responslible for *bla*KPC dissemination in CG258 *K. pneumoniae* strains in Europe and the United States [43].

Some challenges can be found when studying these mobile genetic elements. For instance, the *bla*KPC plasmid pKPC_UVA01, which is widely dispersed in the United States and with strong indications of frequent Tn*4401* transposition in the United States, has been shown to be transmissible but has lower conjugation efficiency *in vitro* than what is observed epidemiologically [44]. More limitations can be observed when using short-read data: in a 5-year clinical study, seventeen random *Klebsiella* strains positive for the presence of *bla*KPC were selected for both short and long-read sequencing. From the analysis of Illumina data, 11 of these 17 strains contained pKPC_UVA01. The PacBio assemblies revealed a pKPC_UVA01-like plasmid in each of these strains, however, only five of these plasmids actually harbored the *bla*KPC gene, while the remaining ones lacked the entire Tn*4401* element, which was present on a different plasmid in the same isolate. Regardless, this demonstrates that plasmid presence (defined by Illumina sequencing) is an unreliable indicator of the mobile unit carrying *bla*KPC. Moreover, long-read data also allowed the detection of Tn*4401* within a Tn2-like element in many different plasmids [45].

1.6.2. OXA-48-like

The OXA-48 enzyme belongs to the class D β-lactamases, which are not inhibited by clavulanic acid and tazobactam. OXA-48-like enzymes are able to hydrolyze all β-lactams and were first identified from a resistant *K. pneumoniae* isolate recovered in Turkey, in 2001 [46], and currently mainly found in *K. pneumoniae* and *E. coli* clinical strains co-producing the CTX-M-15 β-lactamase [47]. The *in vitro* hydrolytic efficiency of OXA-48 against imipenem is tenfold higher than any other oxacillinase [48], however, not all

OXA-48-like enzymes have hydrolytic activity against carbapenems, being OXA-48, OXA-162, OXA-244, OXA-181, and OXA-232 the most commonly found carbapenemases [49].

Recent studies have indicated the transposon Tn*1999* as the main responsible for the mobilization of the *bla*$_{OXA-48}$ gene and its worldwide spread [50]. The Tn*1999*-like transposons are always integrated into the transfer inhibition gene (*tir*) of pOXA-48-like plasmids. Stepwise insertion of other transposons and insertion sequences resulted in the emergence of seven variants of Tn*1999* [51]. The structure of the first Tn*1999* described consists of two copies of an insertion sequence (IS*1999*) flanking the *bla*$_{OXA-48}$ and the *lysR* genes (**Figure 8**). In terms of geographical distribution, OXA-48 is commonly described in the Middle East and North African regions and is commonly associated with the IncL-type plasmids [49]. However, a *K. pneumoniae* isolate was described carrying *bla*$_{OXA-48}$ within an inverted Tn*1999*.1 in an IncM1 plasmid, which was also responsible for within-patient dissemination in the gut microbiota across three Enterobacterales species [52].

However, among the OXA-48-like enzymes, there are different characteristics in terms of mobility and epidemiology. For instance, the OXA-181 β-lactamase, which contains four amino acid substitutions and similar hydrolytic activity when compared with OXA-48 [53], is more predominant in the Indian subcontinent, North Africa and, more recently, Southern Europe [50]. The *bla*$_{OXA-181}$ gene is usually contained on the Tn*2013* transposon, flanked by the insertion sequence IS*Ecp1* and the truncated *lys*R and *ere* genes (**Figure 8**). Although little is known about this transposon's variants, it is frequently found within the ColE, IncX, IncN, and IncT replicon types [53], with a specific 51kb IncX3 being recently reported in multiple countries [54] and transmitted between different *K. pneumoniae* clones [55]. This MGE had also shown the ability to integrate itself within Enterobacterales chromosomes [56], such as the high-risk *K. pneumoniae* ST147 [57].

1.6.3. NDM-like

The New Delhi metallo-β-lactamase 1 (NDM-1) was described as a potential major global health problem since its first discovery in 2008 [58]. This enzyme confers to bacterial species, mainly *K. pneumoniae* and *E. coli*, resistance to all antibiotics except tigecycline and colistin [59]. Several variants of NDM have been reported, mostly from Asia, which results in different transcriptional results and drug affinity [60]. The detection of patterns of *bla*$_{NDM}$ dissemination is particularly hard to achieve, since it is proven that the *in vivo* transfer of *bla*$_{NDM-1}$ can occur between different Enterobacterales species in the same patient [58].

The gene that encodes this carbapenemase is also plasmid-mediated and sometimes found in co-production with another carbapenemase, resulting in a synergistic effect that confers high-level antibiotic resistance [61]. Among the different Inc groups, the *bla*$_{NDM}$ gene can be found including IncC, IncFIB, IncFII, IncHI1B, IncM, IncN, IncQ, IncR, and IncX3 [41].

The *bla*$_{NDM}$ gene is usually found within the Tn*125* transposon (**Figure 8**), flanked upstream by a complete or truncated insertion sequence ISAba125 and downstream by the *ble*$_{MBL}$ gene, which confers resistance to bleomycin, suggesting that the *bla*$_{NDM}$ gene emerged in *Acinetobacter* sp. within the Tn*125* transposon [62]. In Enterobacteraless, the ISAba125 always occurs upstream of this resistance gene, either as a full element, truncated or in association with other insertion sequences [63]. Recently it was

demonstrated that although the Tn*125* did play an essential role in the early plasmid-spread of *bla*$_{NDM}$, it was lately overtaken by other elements such as the Tn*3000* transposon and the IS26-flanked pseudo-composite transposon, which also plays a major role in the genetic reshuffling of *bla*$_{NDM}$ [64].

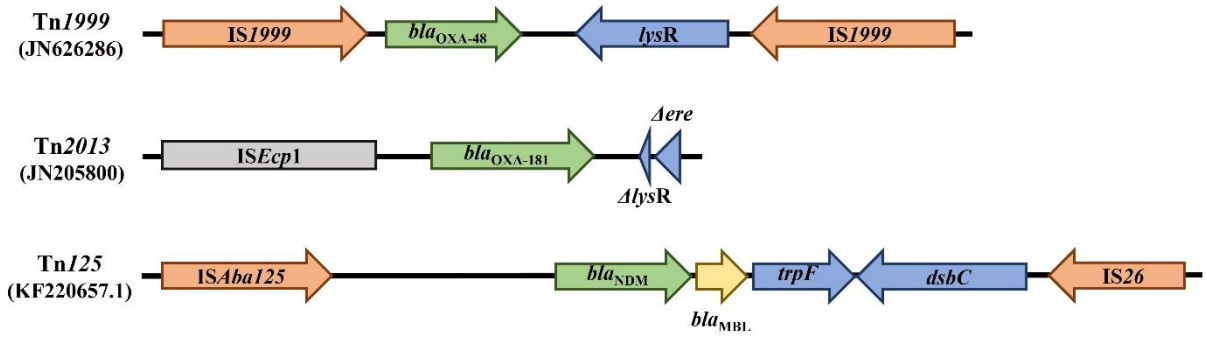

Figure 8 - General scheme of the transposons harbouring the *bla*$_{OXA-48}$, *bla*$_{OXA-181}$ and *bla*$_{NDM}$ genes.

1.6.4. Other carbapenemases

While undoubtedly the KPC and OXA enzymes are the carbapenemases with the highest worldwide spreading rates, and NDM the most representative metallo β-lactamase, especially in the Asian continent, clinical reports of high resistant phenotypes due to other carbapenemases have recently increased. Among these, we highlight the Verone integron-encoded metallo-β-lactamase (VIM), the Imipenemase (IMP), and the Guyana extended-spectrum (GES)-type enzymes [65]. Overall, although the biochemical properties of these carbapenemases are well characterized, the same cannot be observed for the genetic environment of the genes that encode them.

The main variants of *bla*$_{VIM}$ (-1, -2, and -4) have been commonly identified on the class 1 integron in an IncA/C or IncN plasmids in *K. pneumoniae*, but also in other species such as *E. coli*, and *Enterobacter* [66].

Similarly to what was observed with the *bla*$_{VIM}$ gene, the class 1 integrons also appear to be the main responsible for *bla*$_{IMP}$ dissemination among *K. pneumoniae* strains. Among its variants, IMP-4 is the most reported one and, in Asia, is usually found within an IncN plasmid [67]. Furthermore, interspecies transfer of the plasmids harbouring this carbapenemase has already been tested for *Pseudomonas aeruginosa* and *Enterobacter* [68].

The emergence of the GES-like carbapenemases begins with its discovery in an *E. coli* isolate on a class 3 integron and, shortly after, in *K. pneumoniae* [69]. However, the current status quo indicates that the *bla*$_{GES}$-like genes, mainly *bla*$_{GES-1}$ and *bla*$_{GES-5}$, reside often in class 1 integrons found on a considerable variety of plasmids in *K. pneumoniae* clones [70]. It is noteworthy, that GES-like carbapenemases are mostly associated with environmental samples, such as hospital wastewater [71].

1.7. MGE on carbapenemase-producing non-*K. pneumoniae* species

The spreading of the *bla*$_{KPC}$ is also frequently observed in other species of *Klebsiella*. For instance, nosocomial *bla*$_{KPC}$-positive *K. quasipneumoniae* strains retrieved from a hospital setting appeared to have the propensity to take up multiple carbapenemase plasmids from different origins.

Additionally, a rapid genetic rearrangement was observed in the mobile genetic elements carrying *bla*$_{KPC}$ in this species [72]. Across the globe, there are multiple reports of different *K. quasipneumoniae* clinical strains harboring the *bla*$_{KPC}$ gene [73–75]. There has also been an increase of *K. variicola* clinical strains, commonly misidentified as *K. pneumoniae* due to its close phylogenetic relationship [4], encoding the *bla*$_{KPC}$ gene [76,77]. In *K. oxytoca*, IncN type plasmids are commonly found harboring the *bla*$_{KPC-2}$ gene, as well as in other closely related species, such as *K. michiganensis* and *K. grimontii* [78].

The rapid spread of the *bla*$_{KPC-2}$ gene has been linked to the efficiency in the Tn*4401* transposition: in Ecuador, the *bla*$_{KPC-2}$-Tn*4401*a, mainly found in ST307 and ST258 *K. pneumoniae*, was lately spread to *K. oxytoca* and *K. aerogenes*, as well as other Enterobacterales strains [79].

When it comes to interspecies transmission, *in vitro* conjugation experiments have shown that *bla*$_{KPC-2}$ can be transferred with the assistance of the IS26 in a composite transposon and the transfer operon in *E. coli* [80]. In addition, the horizontal transfers of plasmid, mainly from the IncF type, have also strongly contributed to the *bla*$_{KPC}$ dissemination among Enterobacterales species.

Within the *K. oxytoca* species complex, *bla*$_{OXA-48}$ is commonly reported within IncL in hospital outbreaks [78]. However, few case studies were found describing *bla*$_{OXA-48}$ genes in other non-*K. pneumoniae* species.

The identification of the *bla*$_{NDM}$ gene associated with complete or truncated Tn*125* sequences is common in clinical strains of *K. pneumoniae* species complex [81], as well as in *K. michiganensis* [82], and in *K. aerogenes* [83], although most genomic studies came from Asian studies, and little is known about the molecular epidemiology of NDM carbapenemases in Europe. Intriguingly, a *K. oxytoca* harboring a *bla*$_{NDM-1}$-encoding IncC plasmid was reported in a clinical context [84].

In *K. grimontii* and *K. michiganensis*, the *bla*$_{VIM-1}$ gene was detected in an IncHI2 and IncC plasmid, respectively [85]. In Portugal, a *bla*$_{VIM-2}$ gene was found on a plasmid-borne integron in a *K. oxytoca* clinical isolate [86]. Similar to other bacterial hosts, class 1 integrons appeared to play a vital role in the propagation of this carbapenemase gene.

The *bla*$_{IMP-4}$ was reported in similar IncFIB(Mar)/IncHI1B-like plasmids within the *K. pneumoniae* species complex. Moreover, as the *bla*$_{IMP}$ gene is frequently found in co-existence with other carbapenemases, here the *bla*$_{NDM-1}$ gene was also found in the same plasmid [81]. The *bla*$_{IMP}$ was also reported within a class 1 integron in co-production with *bla*$_{KPC-2}$ in *K. quasipneumoniae* [87] and *K. oxytoca* [88]. In fact, while doing this research, numerous cases of class 1 integrons harbouring *bla*$_{IMP}$ genes in *K. oxytoca* clinical strains were observed.

To the best of our knowledge, there are no clinical reports of non-*K. pneumoniae* species expressing the GES carbapenemase, although a recent study has found two distinct plasmids harbouring the *bla*$_{GES-5}$ gene on a class 1 integrons in *K. grimontii* and *K. quasipneumoniae* strains retrieved from hospital effluents [71]

1.8. Goals

Since it is established that carbapenemases are one of the main agents of antibiotic resistance, and most of the genes that encode this class of enzymes are horizontally transferred within and/or between species, the first task of this project was to provide a full disclosure of the main characteristics of the large variety of mobile genetic elements associated with resistance in *Klebsiella* spp.

The main goal of this work is to identify the main mobile genetic elements in carbapenem resistant *Klebsiella* spp. clinical strains and to perform a genetic characterization of these elements in order to better understand their role in the dissemination of carbapenemase resistance genes. Additionally, since little is described on the mobilome of non-*K. pneumoniae*, a greater emphasis on these species will take place.

2. Methodology

2.1. Literature review

A narrative literature review was performed between September 2022 and November 2022, based in the PubMed search engine (https://pubmed.ncbi.nlm.nih.gov/), with no time and language restrictions. The main goal of this review was to get a piece of updated information on mobile genetic elements, which ones can be found in the *Klebsiella* genus and their role on resistance dissemination. Additionally, recent national epidemiological studies were taking into account in order to understand which carbapenemase genes can be commonly found in *Klebsiella* spp. clinical strains, and in which Inc-type plasmids are they usually encoded.

2.2. Hospital centre setting and bacterial strains

A total of 1140 *K. pneumoniae* clinical strains were collected between 2019 and 2022 at three tertiary care hospital centres located in Portugal using standard clinical operating procedures and were sent to the Laboratory of Microbiology Research in Environmental Health of the Faculty of Medicine, Universidade de Lisboa (ULisboa), for advanced molecular analysis. The identification was performed by microbiology laboratories using conventional methods or automated systems such as Vitek2® (BioMérieux, Marcy, l'Étoile, France) or MicroScan (Snap-on, Kenosha, WI, USA). All strains were maintained frozen in BHI broth (VWR Prolabo, Lisbon, Portugal) plus 15% glycerol at −80 °C. For analysis, the strains were grown in BHI broth for 18 h at 37 °C and seeded in Mueller-Hinton (MH) agar (VWR Prolabo, Lisbon, Portugal).

2.2. Phenotypic characterization

The standardized Kirby–Bauer disk diffusion technique was performed for antimicrobial susceptibility testing, in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.[89] The bacteria were placed into MH plates and incubate at 37 ºC for 18 hours. After growth, the strains were suspended in test tubes containing 6.5 mL of distilled water (ADE). With a swab, the bacteria present in the tube are uniformly transferred to a new MH plate. The antibiotic disks were placed on the plate and incubate at 37°C for 18 hours. Detailed instructions for Mueller–Hinton agar medium (VWR Prolabo, Lisbon, Portugal), including preparation and storage, are also available in the same EUCAST guidelines document. Susceptibility was tested for a panel of antibiotics: amoxicillin/clavulanic acid (20/10 µg), cefoxitin (30 µg), cefotaxime (5 µg), ceftazidime (10 µg), imipenem (10 µg), gentamicin (10 µg), ciprofloxacin (5 µg), tigecycline (15 µg), aztreonam (30 µg), ertapenem (10 µg), meropenem (10 µg), and ceftazidime–avibactam (10/4 µg) (Biorad, Algés, Portugal). The strains were categorized as susceptible, standard dosing regimen (S); susceptible, increased exposure (I); and resistant (R) by applying the breakpoints in the phenotypic test results. A complementary ETEST® (BioMérieux, Marcy l' Étoile, France) for ceftazidime/avibactam was also performed for the resistant strains.

The inhibition zones were interpreted according to the EUCAST breakpoint tables for interpretation of minimum inhibitory concentration (MIC) and zone diameters (version 13.0, 2023; available at https://eucast.org/clinical_breakpoints).

Multidrug-resistant (MDR) bacteria were defined as those that acquired nonsusceptibility to at least one agent in three or more antimicrobial categories, in accordance with the United States Centers for Disease Control and Prevention (CDC) and the European Centre for Disease Prevention and Control (ECDC) consensual definition.[91]

2.3. Genotypic characterization

The bacteria were collected from the MH in Eppendorf's containing 1 mL of ADE. The Eppendorf's were placed in water bath for 10 min at 95°C, and then centrifuge at 12,000 rpm for 4 min at 4°C. The supernatant is extracted and stored in a new Eppendorf at -20 ºC.

The amplification step war performed in Polymerase Chain Reactions (PCR) tubes where the ADE, *NZYTaq II Green Master Mix* (NZYTech, Lisbon, Portugal), primers and DNA, before getting into the thermocycler with the respective programme. To confirm the identification of the bacterial strains, the universal 16S rRNA bacterial primers 27F F:5′-AGAGTTTGATCCTGGCTCAG-3′; 1392R R:5′-GGTTACCTTGTTACGACTT -3′ were used, and the detection of carbapenemase genes was guaranteed with primers designed for $bla_{\text{OXA-48}}$ F:5′-GGCTGTGTTTTTGGTGGCATC-3′; R:5′-GCAGCCCTAAACCATCCGATG-3′, $bla_{\text{KPC,}}$[92] $bla_{\text{VIM,}}$[93] $bla_{\text{NDM,}}$[94] and $bla_{\text{GES.}}$[95]

For visualization, 5 µL of each PCR product ran for approximately 45 min at 100V and 200mA in a 150 mL 1% agarose gel made with 135mL ADE, and 15mL 10× concentrated Tris-Borate-EDTA (TBE buffer) (NZYTech, Lisbon, Portugal). For staining, 5.5 µL of *GreenSafe Premium* (NZYTech, Lisbon, Portugal) was added to the gel. The PCR assays included positive and negative controls.

Are amplified 16S sequences and carbapenemase genes were purified using the ExoCleanUp FAST (VWR Prolabo®, Lisboa, Portugal) kit and sequenced at STABVida Portugal.

The BLAST program, available at the National Centre for Biotechnology Information website (http://www.ncbi.nim.nih.gov) was used to search for nucleotide sequences. Multiple-sequence alignments were performed with the Clustal Omega program, available at (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.4. Whole Genome Sequencing (WGS)

The collection used for whole genome sequencing (WGS) analysis represent a total of 218 nonduplicate strains, based on their phenotypic and genotypic resistance determinants previously accessed by antimicrobial susceptibility test and PCR-based screening for carbapenemases genes, respectively.

The genomic DNA was extracted for WGS from cultures grown overnight in Mueller–Hinton agar, using the NZY Tissue gDNA Isolation kit (NZYTech, Lisboa, Portugal), as per the manufacturer's recommendations and sent to STABVida Portugal for sequencing. Indexed libraries were prepared using the KAPA HyperPrep Library Preparation Kit (Roche, Switzerland), according to the manufacturer's recommended protocol and the sequence was performed using an Illumina HiSeq Novaseq 6000

platform with paired end reads (2 x 151 bp). The raw data quality control was performed using FASTQC v0.11.9, and the trimming and de novo assembly was performed using CLC Genomics Workbench 12.0.3 (QIAGEN, Aarhus, Denmark). All assemblies were carried out with automatic word size, similarity fraction of 0.95, a length fraction of 0.95 and a minimum contig size of 500 bp.

Antimicrobial resistance, K and O antigen loci, and the Multilocus Sequence Typing (MLST) were identified using Kleborate, a genomic typing tool specific to *K. pneumoniae.*[96] The MLST for *K. aerogenes* strains was performed by analysing the internal fragments of seven housekeeping genes (*dna*A, *fus*A, *gyr*B, *leu*S, *pry*G, *rpl*B, and *rpo*B), the whole genome sequence of these bacterial strains was submitted to the MLST database (available at: https://pubmlst.org/) for allele and sequence type attribution.

Early plasmid analyses and identification of incompatibility groups will be performed through the PlasmidFinder platform with the following cut-off values: minimum of 60% coverage and 95% identity (https://cge.food.dtu.dk/services/PlasmidFinder/).

Graphical visualization of the WGS data was obtained through GraphPad Prism version 8.0.2 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com.

2.5. Mobilome analysis for clinical significative impact strains

A total of 19 plasmids from 16 strains of distinct species, hospitals of origin, lineages, and resistance genes, were selected for reconstruction and analysis of their genomic features. The MobileElementFinder online tool (https://cge.food.dtu.dk/services/MobileElementFinder/) was used to identify mobile genetic elements that may carry resistant determinants and in which contig they are encoded [97]. The contigs of interest were submitted to the BLAST program in the search for a reference plasmid. With mlplasmid v2.1.0 (https://sarredondo.shinyapps.io/mlplasmids/), it was possible to filter for plasmid-origin contigs, as it allows to distinguish sequenced contigs that derived from plasmids and the ones that derived from the chromosome[98]. Lastly, the BLAST Ring Image Generator (BRIG) was used for a visualization and circular comparisons of resistance plasmids [99]

2.6. *bla*$_{KPC-98}$ gene cloning in pCR4-TOPO TA vector and expressed in TOP10 *E. coli* cells

In order to evaluate whether the novel KPC-3 variant, KPC-98, confer resistance to CZA, the *bla*$_{KPC-98}$ gene and its promoter were amplified by PCR with primers previously described (9), using NZYTaq II 2x Green Master Mix and ligated into the pCR™4-TOPO™ vector (ThermoFisher Scientific®) following the manufacturer's instructions. Ligation mixtures were introduced by chemical transformation into TOP10 chemically competent *E. coli* cells (ThermoFisher Scientific®). Transformants were selected on Luria-Beranti (LB) agar plates containing ampicillin (50 mg/mL) and screened for the presence of the *bla*$_{KPC-98}$ gene by PCR. CZA/IPM zone diameter and CZA/MEM MICs were performed for positive recombinant clones by disk diffusion test and ETEST®, respectively.

2.7. Plasmid stability assessment

A single colony of the KPC-98-producing *K. pneumoniae* (FMUL503) and the KPC-3-producing *K. pneumoniae* (FMUL920; control) were picked up from the freshly streaked agar plate and inoculated into fresh LB broth. Serial culture of each isolate was performed for 10 days, in which 10 µL of the bacterial suspension was transferred to 10 mL of fresh LB broth every 12 hours (equivalent to 10 generations each). The plasmid stability was assessed by streaking each subculture on fresh LB agar plates and randomly selecting three single colonies for PCR detection of the bla_{KPC} gene of the respective resistance plasmids. In the final day of the protocol, a disk diffusion test for CZA was applied for the KPC-98 producing strain.

3. Results

3.1. Phenotypic characterization

The vast majority of this study's bacterial strains presented MDR profile (98.9%; 1127/1140). The susceptibility rates were very low for most antibiotics (**Table 1**

), particularly amoxicillin-clavulanic acid (0.9%), ertapenem (2.6%), ceftazidime (3.5%), aztreonam (4%), cefotaxime (4.6%), and meropenem (5.8%). These rates were also low for imipenem (10%), cefoxitin (14.8%), ciprofloxacin (16.7%), and gentamicin (22.3%). Only exhibited great susceptibility rates were reported for tigecycline (69%) and the novel antibiotic combination, ceftazidime-avibactam (91.4%).

In addition, 24 strains that showed resistance against ceftazidime-avibactam were submitted to an etest. From these, 13 (54.2%) showed resistance against this antibiotic according to the EUCAST guidelines (>8 µg/mL).

Antibiotic tested	AST Dose (µg)	Interpretation[a] number of strains (n=1140)			Susceptibility Rate
		S	I	R	
Penicillins:					
Amoxicillin-clavulanic acid	20/10	10	-	1130	0.9%
Cephalosporins:					
Cefoxitin	30	169	-	971	14.8%
Cefotaxime	5	53	18	1069	4.6%
Ceftazidime	10	40	25	1075	3.5%
Ceftazidime-avibactam	10/4	1042	-	98	91.4%
Carbapenems:					
Imipenem	10	114	93	933	10.0%
Ertapenem	10	30	-	1110	2.6%
Meropenem	10	66	236	838	5.8%
Monobactams:					
Aztreonam	30	46	22	1072	4.0%
Fluoroquinolones:					
Ciprofloxacin	5	190	101	849	16.7%
Aminoglycosides:					
Gentamicin	10	254	-	886	22.3%
Tetracycline:					
Tigecycline	15	787	-	353	69.0%

[a]Following European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (version 13.0, 2023; available at https://eucast.org/clinical_breakpoints). Abbreviations: S, susceptible; I, susceptible, increased exposure; R, resistant.

Table 1 - Disk diffusion test results for 1140 clinical *Klebsiella* spp. strains, Portugal, 2019–2022.

3.2. Genotypic characterization

3.2.1. 16S identification of the bacterial strains

A total of 1140 clinical strains were collected between 2019 and 2022 at hospital settings, then sent to the Laboratory of Microbiology Research in Environmental Health of the Faculty of Medicine, Universidade de Lisboa (ULisboa), for advanced molecular analysis. All bacterial strains identified via 16S rRNA gene amplification, pointing *K. pneumoniae* as the most predominant species (93.3%; 1064/1140), followed by *K. aerogenes* (3.7%; 42/1140), and *K. quasipneumoniae* (0.9%; 10/1140). Other Species detected included *K. oxytoca* (0.5%; 6/1140), *K. variicola* (0.4%; 5/1140), *K. michiganensis* (3/1140; 0.3%), a *K. grimontii* isolate (1/1140; 0.1%), as well as some unidentified *Klebsiella* species (3/1140; 0.3%). Some *Raoultella* strains (0.5%; 6/1140) were also found. Phylogenetic analyses have shown the *Raoultella* genus nested in the *Klebsiella* genus, being proposed this genus to be abandoned and considered a junior synonym of *Klebsiella* [100]. For this reason, these *Raoultella* strains will be taking into account for this study.

3.2.2. Carbapenemase genes

Carbapenemase production was detected in most bacterial strains when screening their genes by PCR (**Figure 9**). In fact, only a small percentage (20.1%; 229/1140) of the strains included in this study did not exhibit any carbapenemase encoded in their genome or within an MGE. The *bla*$_{KPC-3}$ gene was undoubtably the most frequently found gene, followed by *bla*$_{OXA-181}$ and *bla*$_{NDM-1}$. Co-production of two types of carbapenemases was detected in 50 strains.

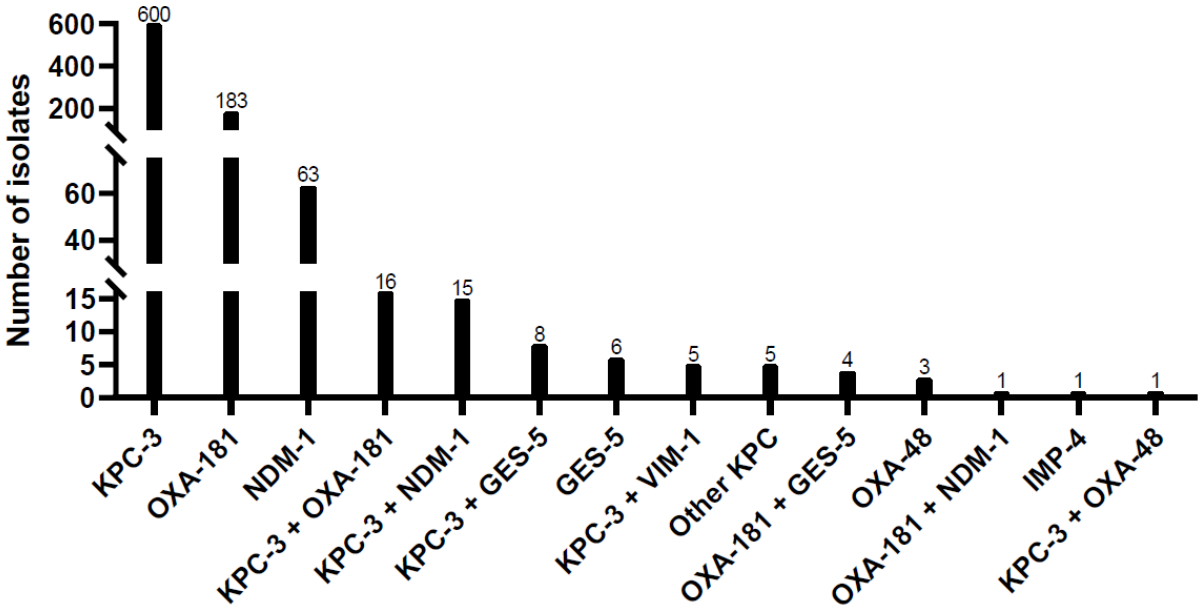

Figure 9 - Carbapenemase genes identification in 1140 clinical *Klebsiella* strains, Portugal, 2019–2022.

3.3. Whole Genome Sequencing

3.3.1. Bacterial identification

Whole genome data showed, overall, good concordance with the identification provided by the amplification of the 16S rRNA gene of the strains selected for in-depth genomic analysis. *K. pneumoniae* is the most representative species among this group (83.9%; 183/218), followed by *K. aerogenes* (7.8%; 17/218). *K. quasipneumoniae* (2.3%; 5/218), *K. michiganensis* (2.3%; 4/218), *K. oxytoca*, *K. variicola* (1.4%; 3/218), and a *K. pasteurii* isolate (0.5%; 1/218) were also correctly identified. No *K. grimontii* was detected by the Kleborate, as it was presumably misidentified by PCR. Two of the *Raoultella* strain was identified as *K. ornithinolytica*, a homotypic synonym of *Raoultella ornithinolytica* [101]. This consistency of results between the 16S gene amplification and the whole genome data, demonstrates that previous identification of bacterial species by PCR is still a reliable and quick tool for the modern microbiologist.

Due to these results, and for practical purposes, the strains in this study were grouped according to their species complex for further genomic analysis:

(i). *K. pneumoniae* species complex [4] (N=191): consisting of all *K. pneumoniae* (n=183), *K. quasipneumoniae* (n=5), and *K. variicola* (n=3) strains;

(ii). *K. oxytoca* species complex [78] (N=10): consisting of all *K. oxytoca* (n=3), and *K. michiganensis* (n=4) strains, *K. pasteurii* (n=1), in addition to the *K. ornithinolytica* strains (n=2), as it is closely related with the *K. oxytoca* species complex;

(iii). *K. aerogenes* strains (N = 17).

3.3.2. Epidemiological markers: MLST, capsular- and O antigen- loci

The multi locus sequence typing (MLST) is a procedure for the characterization of bacterial strains using the sequences of internal fragments of usually seven house-keeping genes [102]. Different species require different MLST schemes and, to the date, the PubMLST database (https://pubmlst.org/) has provided clonal schemes for *K. pneumoniae* (*gapA, infB, mdh, pgi, phoE, rpoB*, and *tonB*), *K. aerogenes* (*dnaA, fusA, gyrB, leuS, pryG, rplB*, and *rpoB*) and *K. oxytoca* (*gapA, infB, mdh, pgi, phoE, rpoB*, and *tonB*).

The synthesis and export machinery of the *Klebsiella* polysaccharide capsule are encoded in a single 10-30 kbp region of the genome known as the K locus. Likewise, the key determinants of the O antigenic polysaccharide are located at the O locus. Both the polysaccharide and the O antigen had previously shown potential for serological typing of *Klebsiella* strains, however single nucleotide polymorphisms are hard to detect by this method and many strains are serologically nontippable, thus the need for sequencing and identification of K and O loci for serotype prediction and epidemiological marker, in order to implementation of control strategies targeting the capsules or lipopolysaccharides of *Klebsiella* species.

The combination of these three epidemiological markers was applied for an in-depth characterization of the *Klebsiella* populations in this study. For the *K. pneumoniae* strains, it was detected a large diversity of clones, although clear predominance of ST147-KL64-O2 (34/191; 17.8%) and ST17-KL25-O5 (31/191; 16.2%) was detected, as well as two ST13 populations (32/191; 16.8%).

Noteworthy, a great number of ST11-KL105-O2 *K. pneumoniae* is presented, due to an outbreak study previously published by the research group [103]. The distribution of the main clones is not identical between the three hospitals included in this study (**Figure 10**). The *K. quasipneumoniae* and *K. variicola* strains included in this group presented distinct lineage types from those described in K. pneumoniae, with two *K. quasipneumoniae* sharing the same lineage (ST414-KL123-O3)

Moreover, a total of 17 *K. aerogenes* strains of were identified in a single hospital. In this species, there's a clear predominance of ST93-O5 (76.5%; 13/17), commonly referenced within the *K. aerogenes* clonal complex 3 (CC3), as the most frequently found MLST profile. The Kleborate software was unable to accurately identify the K locus of the *K. aerogenes* strains, although it pointed KL68 as "closest match" for most of the strains.

The *K. oxytoca* species group revealed great diversity of species and epidemiological markers, although we detected three ST2-KL74 *K. oxytoca* clones (3/10; 30%) in the same hospital during different timelines, two of them carrying the OL104 locus and the other one encoding the O3 antigen type.

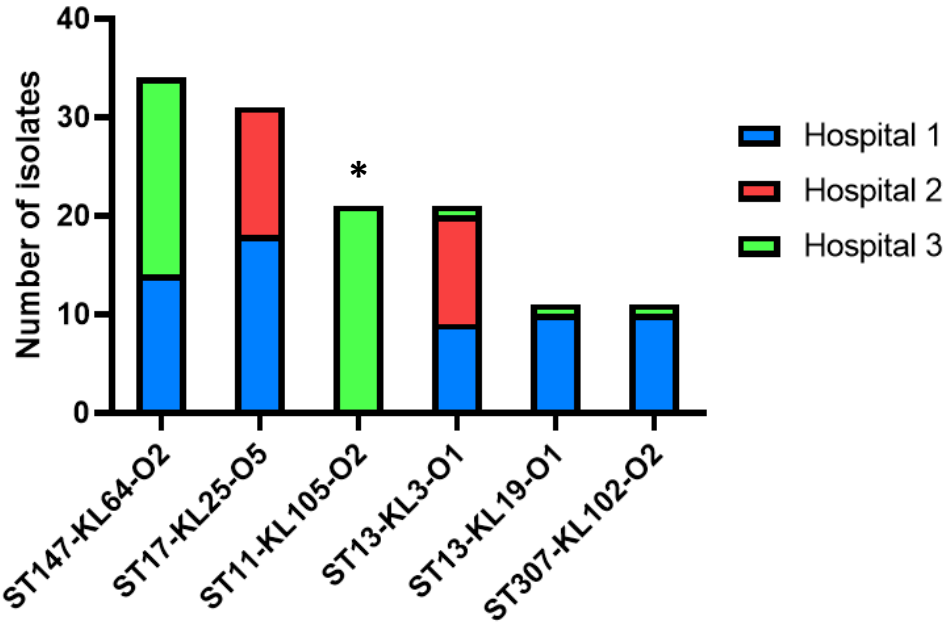

Figure 10 - Distribution of main *K. pneumoniae* complex clones in Portugal by hospital, 2019–2022. *A great number of ST11-KL105-O2 strains is presented due to an outbreak occurred in 2020.

3.3.3. Carbapenemase genes

The presence of carbapenemase genes was confirmed by the whole genome data. Carbapenemase production was observed in a great number of strains (87.6%; 191/218), however there were significant differences from species to species.

The *bla*$_{KPC-3}$ gene (52.4%; 100/191) was undoubtably the most frequently found gene in the *K. pneumoniae* species complex group (including most of the *K. quasipneumoniae* and *K. variicola* strains), followed by *bla*$_{OXA-181}$ (17.8%; 34/191) and *bla*$_{NDM-1}$ (11.5%; 22/191). Co-production of two different types of carbapenemases was detected in 6 strains (3.1%; 6/191) (**Figure 11**).

The *bla*$_{OXA-181}$ was found in most of the *K. aerogenes* strains (58.8%; 10/17). Furthermore, the *bla*$_{KPC-3}$, *bla*$_{GES-5}$ gene, and the co-production of these two genes (5.9%; 1/17) were also detected in this species.

While the *bla*$_{KPC-3}$ (50%; 5/10) gene was present in *K. michiganensis*, *K. pasteurii*, and *K. ornitholytica* strains, all *K. oxytoca* strains presented the *bla*$_{OXA-48}$ gene (30%; 3/10). Additionally in this group, co-production of *bla*$_{OXA-181}$ and *bla*$_{KPC-3}$ (10%; 1/10) was detected in a *K. michiganensis* bacterial isolate.

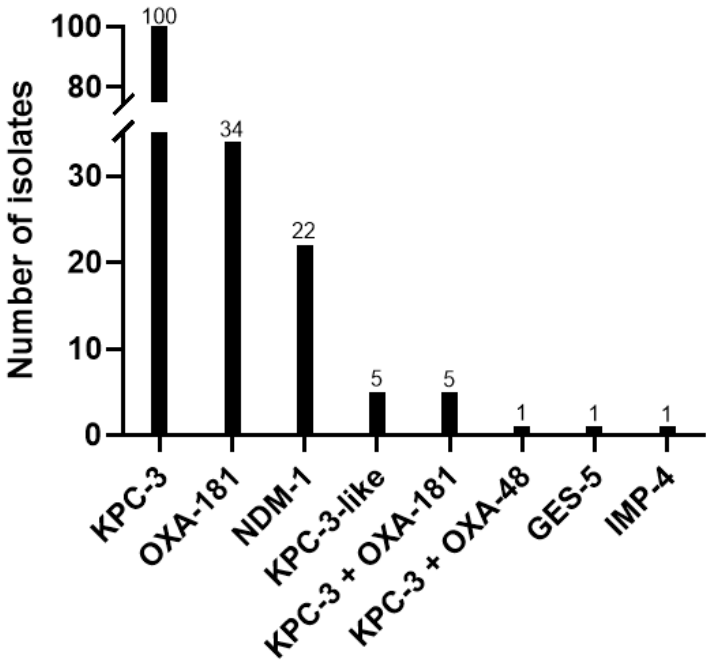

Figure 11 - Carbapenemase genes detected via WGS of 191 clinical *K. pneumoniae* species complex strains, Portugal, 2019–2022.

3.4. Mobilome

3.4.1. Inc families of plasmids

Some similarities were detected when identifying the major plasmid families across the three different (**Figure 12**). Great diversity of plasmid types was found among the *K. pneumoniae* species complex group: IncF-, Col-, and IncR-like plasmids were the most represented types. IncFIB, and Col-like were frequently found in *K. aerogenes* species, which overall presented low plasmid diversity. IncFIB and IncFII were predominant among the *K. oxytoca* species complex group.

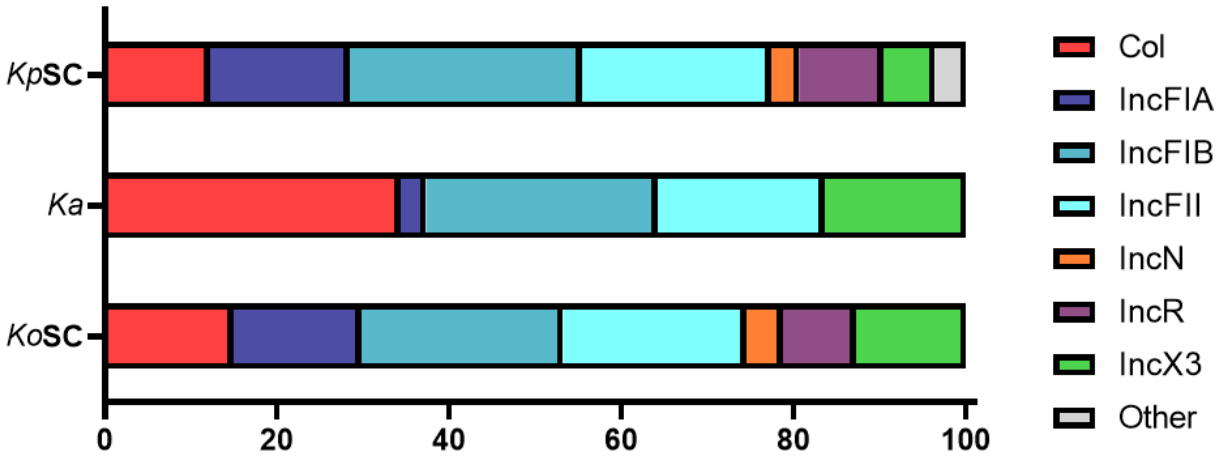

Figure 12 - Major plasmid families relative distribution across the three main *Klebsiella* species groups. Abbreviations: *Kp*SC, *K. pneumoniae* species complex; *Ka*, *K. aerogenes*, *Ko*SC, *K. oxytoca* species complex.

3.4.2. Structural analysis of carbapenemase-encoding plasmids

The following strains were selected for reconstruction and analysis of carbapenemase-encoding plasmids of distinct species, hospital of origin, lineages, and resistance genes (**Table 2**).

Table 2 – List of carbapenemase-producing *Klebsiella* strains selected for plasmid reconstruction and analysis (n=16).

Group	Species	FMUL	Hospital	Lineage	Carbapenemase	BLAST match
*Kp*SC	*K. pneumoniae*	94	1	ST13-KL3-O1	KPC-70	pNY9_2 (CP015387.1)
*Kp*SC	*K. pneumoniae*	424	3	ST11-KL105-O2	NDM-1	pNDM1_LL34 (CP025965.2)
*Kp*SC	*K. pneumoniae*	460	1	ST13-KL19-O1	KPC-3	pNY9_2 (CP015387.1)
					OXA-181	p010_B-OXA181 (CP048332.1)
*Kp*SC	*K. quasipneumoniae*	475	1	ST257-O3	KPC-3	pNY9_2 (CP015387.1)
*Kp*SC	*K. pneumoniae*	503	1	ST13-KL19-O1	KPC-98	pNY9_2 (CP015387.1)
*Kp*SC	*K. pneumoniae*	572	3	ST147-KL64-O2	KPC-3	pWI1-KPC3 (LT838197.1)
*Kp*SC	*K. variicola*	634	1	ST347-KL34-O3	KPC-3	pNY9_2 (CP015387.1)
*Kp*SC	*K. pneumoniae*	672	2	ST13-KL3-O1	KPC-40	pNY9_2 (CP015387.1)
*Kp*SC	*K. pneumoniae*	703	2	ST17-KL25-O5	OXA-181	p010_B-OXA181 (CP048332.1)
*Kp*SC	*K. pneumoniae*	705	2	ST13-KL3-O1	KPC-3	pNY9_2 (CP015387.1)
*Kp*SC	*K. pneumoniae*	828	1	ST20-KL28-O1	IMP-4	pC52_003 (CP042548.1)
Ka	*K. aerogenes*	15	1	ST93-KL68-O5	OXA-181	p010_B-OXA181 (CP048332.1)
Ka	*K. aerogenes*	342	1	ST93-KL68-O5	KPC-3	pNY9_2 (CP015387.1)
					GES-5	pKP-M1144 (MT720902.1)
*Ko*SC	*K. michiganensis*	146	1	ST88-KL66-O1	KPC-3	pNY9_2 (CP015387.1)
*Ko*SC	*K. michiganensis*	277	1	ST330-KL74-O1	KPC-3	pNY9_2 (CP015387.1)
					OXA-181	p010_B-OXA181 (CP048332.1)
*Ko*SC	*K. oxytoca*	376	1	ST2-KL74-O3	OXA-48	p2018C08-141-66k (CP129735.1)

With exception of FMUL572, all KPC-encoding plasmids in this study share notable resemblances with the large 140kb IncF pNY9_2 plasmid, despite being found in all three species groups, distinct lineages and two different hospitals (**Figure 13**). This large plasmid is frequently found carrying other drug resistant genes. On the other hand the pKPC3_FMUL572 plasmid, found in a ST147-KL64-O2 *K. pneumoniae* from Hospital 3, appears to be smaller and carrying the *bla*$_{KPC-3}$ gene as the only resistant determinant (**Figure 14**). Nevertheless, all *bla*$_{KPC}$ genes in these eleven plasmids, were found within the Tn*4401* isoform d transposon.

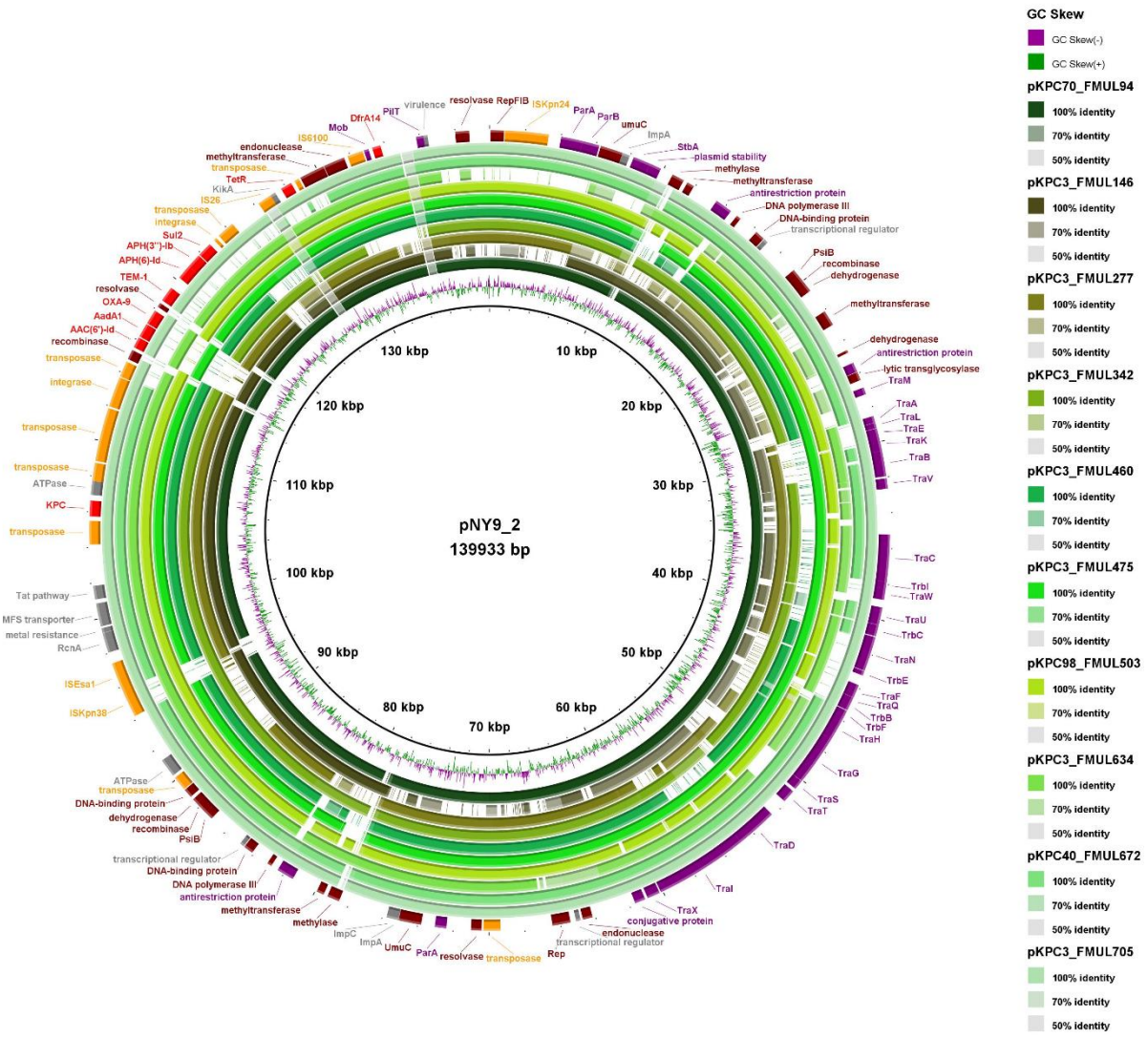

Figure 13 - Plasmid alignment between reference assembled *K. pneumoniae* pKPC70_FMUL94, *K. michiganensis* pKPC3_FMUL146, *K. michiganensis* pKPC3_FMUL277, *K. aerogenes* pKPC3_FMUL342, *K. pneumoniae* pKPC3_FMUL460, *K. quasipneumoniae* pKPC3_FMUL475, *K. pneumoniae* pKPC98_FMUL503, *K. variicola* pKPC3_FMUL634, *K. pneumoniae* pKPC40_FMUL672, and *K. pneumoniae* pKPC3_FMUL705, and plasmid pNY9 (GenBank acc. CP015387), used as backbone plasmid reference. Genes are represented by colored blocks: Red – antibiotic resistance; Orange – transposases; Maroon – DNA replication and modification; Purple – plasmid maintenance and DNA transfer; Gray – other proteins.

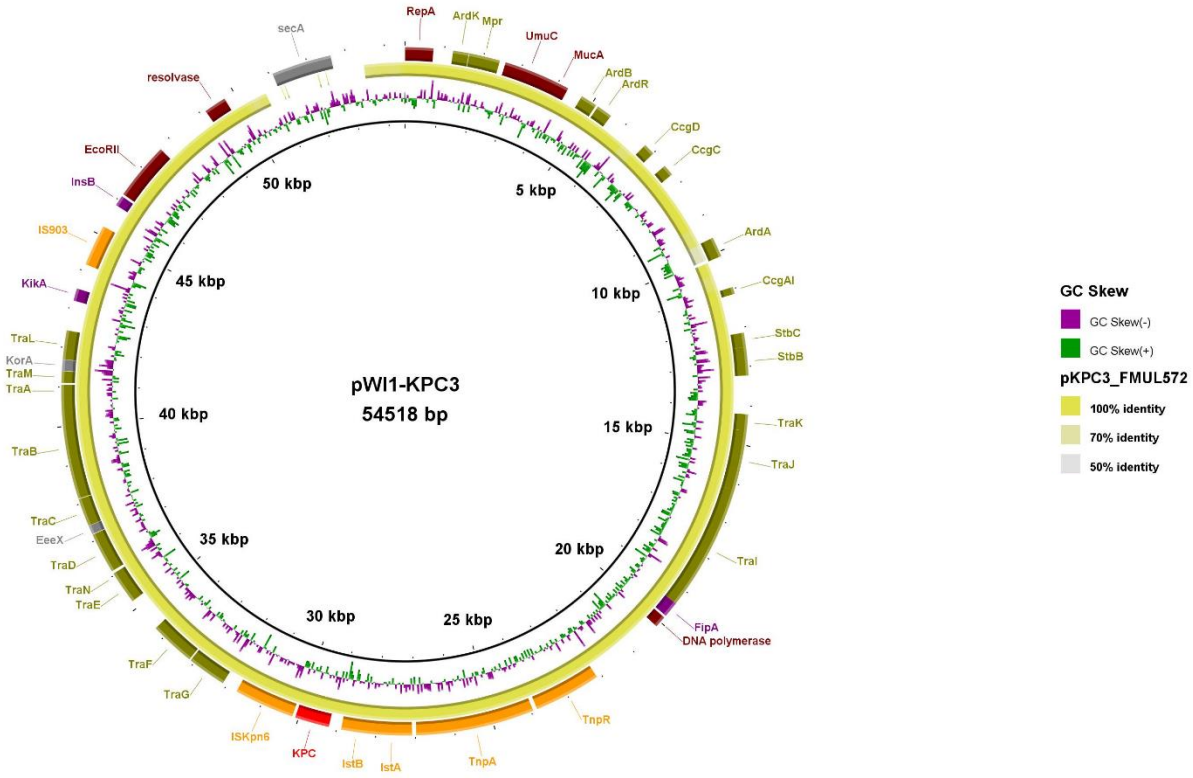

Figure 14 - Plasmid alignment between reference assembled *K. pneumoniae* pKPC3_FMUL572, and plasmid pWI1-KPC3 (GenBank acc. LT838197.1), used as backbone plasmid reference. Genes are represented by colored blocks: Red – antibiotic resistance; Orange – transposases; Maroon – DNA replication and modification; Purple – transcriptional regulation; Olive – plasmid maintenance and DNA transfer; Gray – other proteins.

All the OXA-181-producing plasmids shared great similarities with the 50kb p010_B-OXA181 plasmid, despite being found in three different groups of species (**Figure 16**). The *bla*$_{\text{OXA-181}}$ gene was found upstream the IS*Kpn19* insertion sequence, and within a composite transposon where the quinolone resistance *qnrS1* gene can also be found. This composite transposon shared approximately 70% resemblance with the IS26 transposon, the precise identification of this MGE couldn't be extracted. Furthermore, the pOXA48_FMUL376 plasmid, found in a ST2-KL74-O3 *K. oxytoca*, shared an almost perfect match with a different plasmid: p2018C08-141-66k (**Figure 15**). In this particular case, the *bla*$_{\text{OXA-48}}$ gene is the single resistant determinant found in the plasmid and appears to be within a composite transposon. However, the association between *bla*$_{\text{OXA-48}}$ and this type of MGE is not clear.

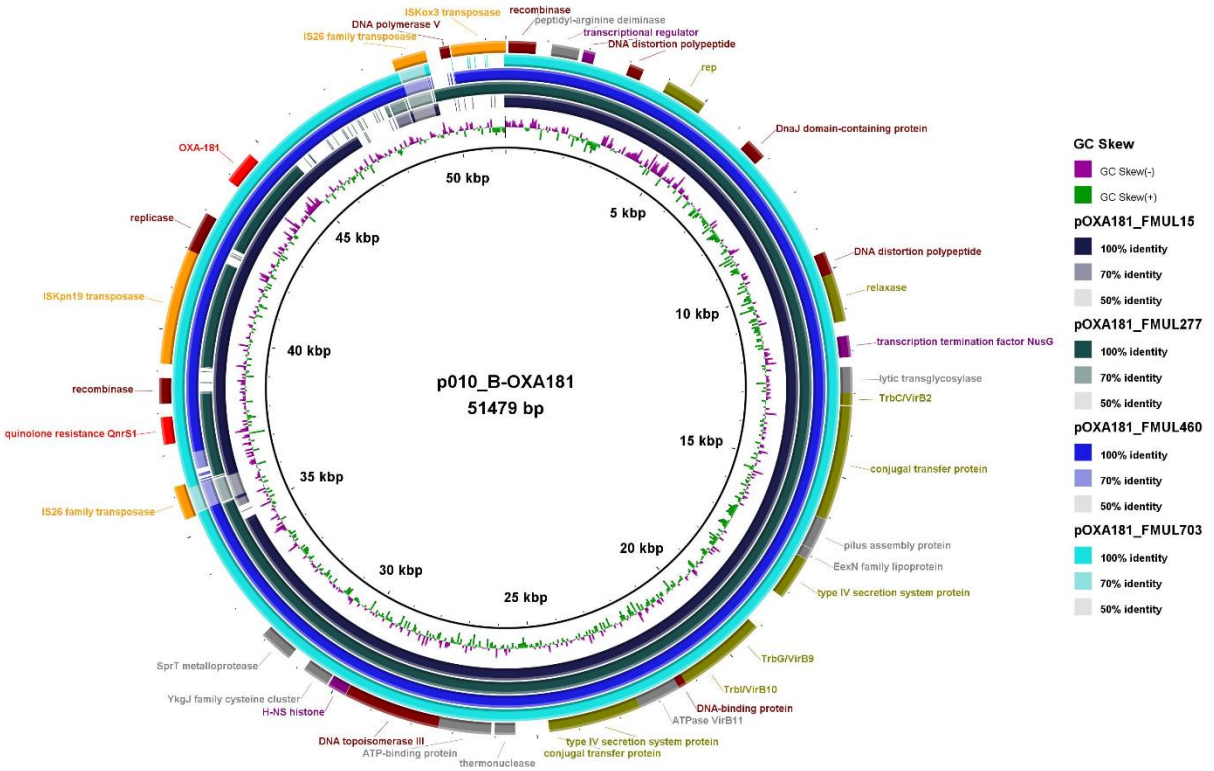

Figure 16 - Plasmid alignment between reference assembled *K. aerogenes* pOXA181_FMUL15, *K. michiganensis* pOXA181_FMUL277, *K. pneumoniae* pOXA181_FMUL460, and *K. pneumoniae* pKPC181_FMUL703, and plasmid p010_B-OXA181 (GenBank acc. CP048332.1), used as backbone plasmid reference. Genes are represented by colored blocks: Red – antibiotic resistance; Orange – transposases; Maroon – DNA replication and modification; Purple – transcriptional regulation; Olive – plasmid maintenance and DNA transfer; Gray – other proteins.

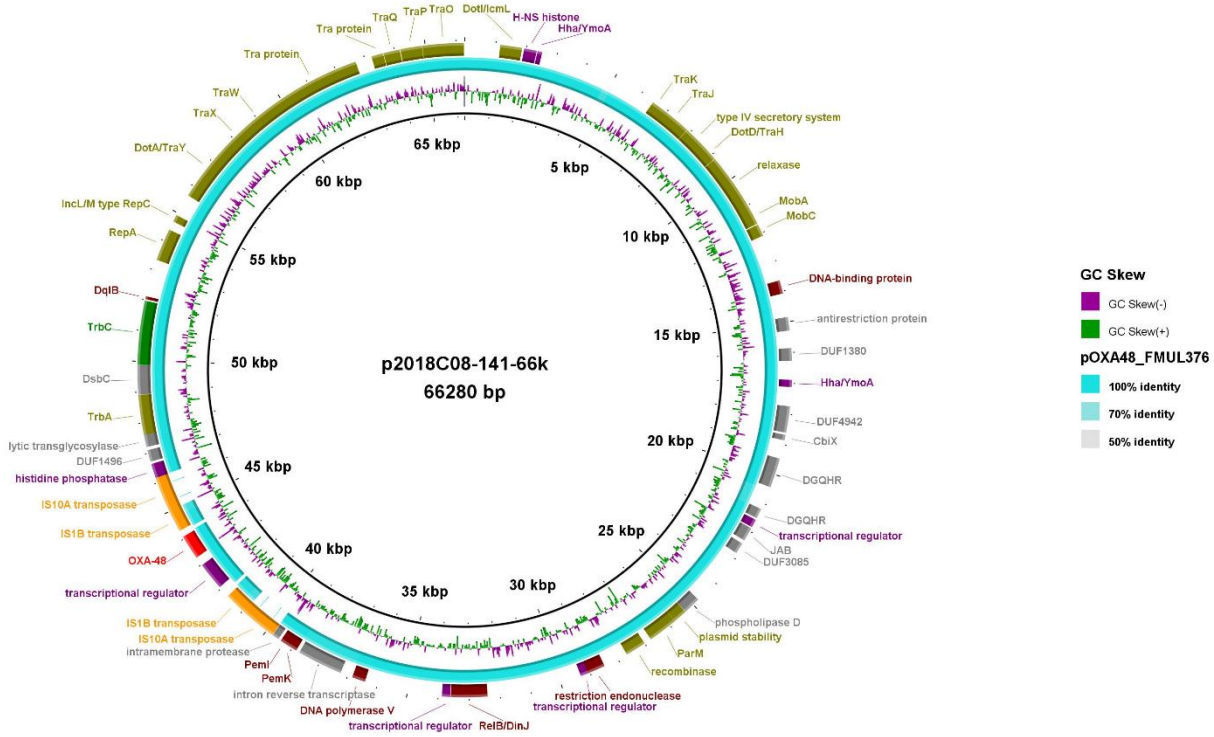

Figure 15 - Plasmid alignment between reference assembled *K. oxytoca* pOXA48_FMUL376, and plasmid p2018C08-141-66k (GenBank acc. CP129735.1), used as backbone plasmid reference. Genes are represented by colored blocks: Red – antibiotic resistance; Orange – transposases; Maroon – DNA binding and modification; Purple – transcriptional regulation and nucleoid-associated proteins; Olive – plasmid maintenance and DNA transfer; Gray – other proteins.

In 2020, an outbreak of NDM-1-producing *K. pneumoniae* ST11-KL105-O2 was reported. Many defects were found during the reconstruction of the pNDM1_FMUL424 plasmid (**Figure 17**) found during the outbreak. Although it is difficult to determine the plasmid size and which MGE may be in association with the *bla*$_{NDM-1}$ gene, elements of the Tn*125* were found, namely an IS26-like transposase (approximately 70% resemblance), and the bleomycin resistance gene, *ble*$_{MBL}$, next to the carbapenemase gene of interest.

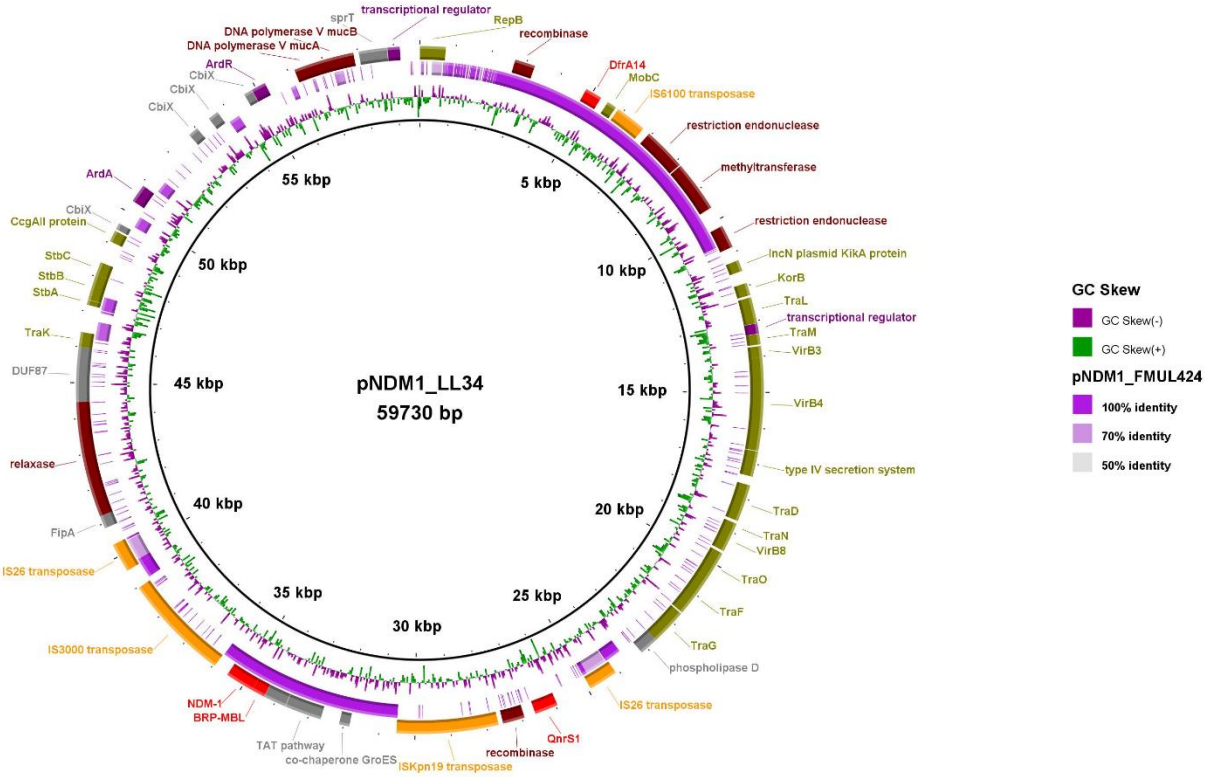

Figure 17 - Plasmid alignment between reference assembled *K. pneumoniae* pNDM1_FMUL424, and plasmid pNDM1-LL34 (GenBank acc. CP025965.2), used as backbone plasmid reference. Genes are represented by colored blocks: Red – antibiotic resistance; Orange – transposases; Maroon – DNA replication and modification; Purple – transcriptional regulation; Olive – plasmid maintenance and DNA transfer; Gray – other proteins.

One ST93-KL68-O5 *K. aerogenes* isolate presented co-production of KPC-3 and GES-5 in different plasmids. The *bla*$_{GES-5}$ gene was found in a small plasmid with little over 10kb (**Figure 18**). Upstream the Tn*5403* transposon the *bla*$_{GES-5}$ gene can be detected in addition with two other resistance genes (*bla*$_{BEL-1}$, and *aac(6')-Ib*).

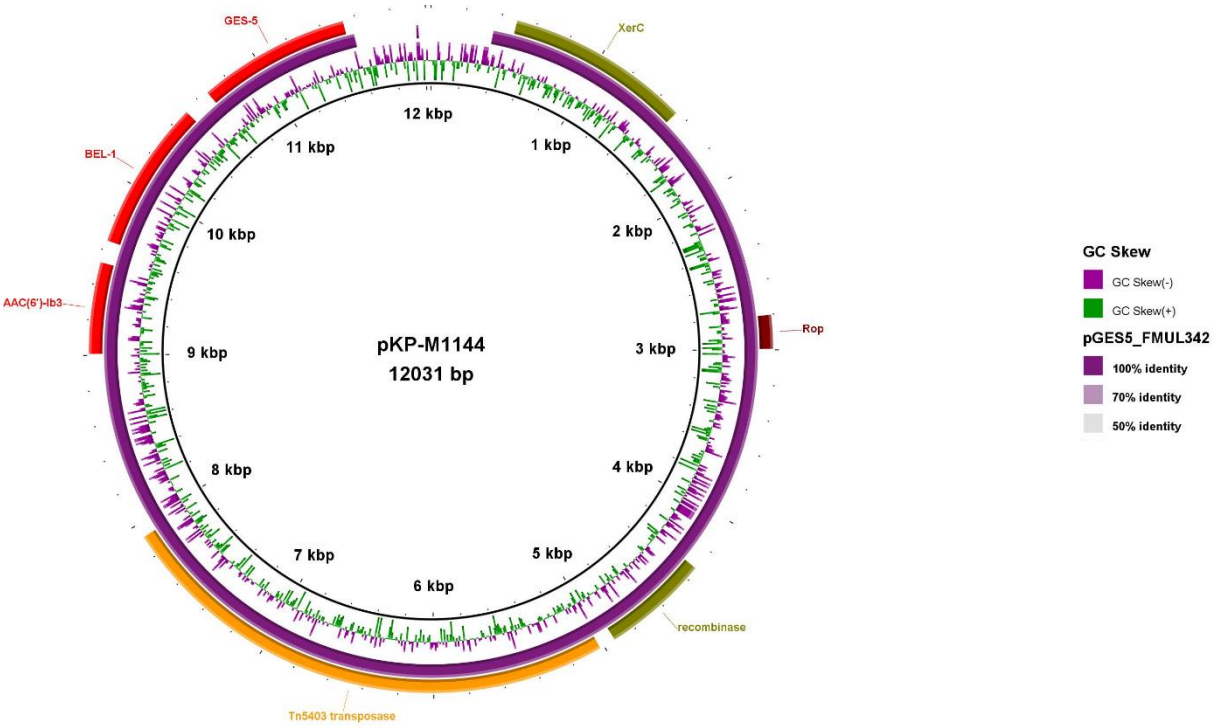

Figure 18 - Plasmid alignment between reference assembled *K. aerogenes* pGES5_FMUL342, and plasmid pKP-M1144 (GenBank acc. MT720902.1), used as backbone plasmid reference. Genes are represented by colored blocks: Red – antibiotic resistance; Orange – transposase; Maroon – regulatory protein; Olive – recombinases.

The reconstruction of the ST20-KL28-O1 *K. pneumoniae* plasmid encoding the *bla*$_{IMP-4}$ gene was unsuccessful (**Figure 19**), although it can be observed to be part of what seems to be a resistant gene cassette with other drug resistance determinants, which is characteristic of class 1 integrons.

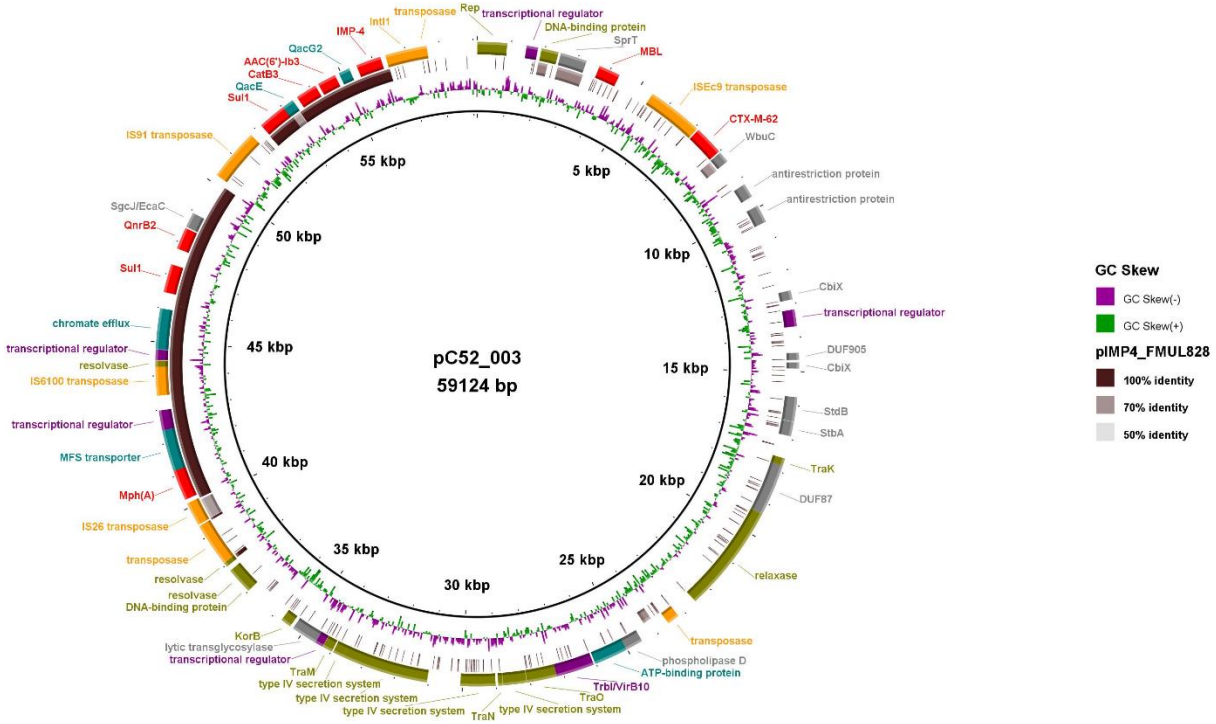

Figure 19 - Plasmid alignment between reference assembled *K. pneumoniae* pIMP4_FMUL828, and plasmid pC52_003 (GenBank acc. CP042548.1), used as backbone plasmid reference. Genes are represented by colored blocks: Red – antibiotic resistance; Orange – transposases and integrases; Teal – transporters; Purple – transcriptional regulators; Olive – DNA binding and transfer; Gray – other proteins.

3.4.3. Case report: novel KPC-98 conferring resistance to CZA

To investigate the phenotypic properties of *bla*$_{KPC-98}$, the gene and its respective promoter were amplified by PCR, ligated into the pCR™4-TOPO™ vector and expressed in TOP10 *E. coli* recipient cells. The recombinants tested positive for *bla*$_{KPC}$ gene following PCR amplification. The positive recombinants acquire resistance to CZA but remain susceptible to carbapenems (**Table 3**).

Table 3 - Antimicrobial susceptibility profile of KPC-98-producing *K. pneumoniae* strains and TOP10 *E. coli* cells containing the *bla*$_{KPC-98}$ cloned into pCR4-TOPO TA vector [a].

Antibiotic	Disk diffusion test (*K. pneumoniae*)		Minimum Inhibitory Concentration (*K. pneumoniae*)		Disk diffusion test (TOP10 *E. coli* + pCR4-TOPO TA vector)		Minimum Inhibitory Concentration (TOP10 *E. coli* + pCR4-TOPO TA vector)	
	Inhibition zone (mm)	Interpretation	MIC (µg/mL)	Interpretation	Inhibition zone (mm)	Interpretation	MIC (µg/mL)	Interpretation
CZA	10	R	24	R	12	R	192	R
IPM	12	R	6	R	27	S	-	-
ETP	14	R	0.06	S	-	-	-	-
MEM	13	R	0.38	S	-	-	0.19	S

[a]Following European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (version 13.0, 2023; available at https://eucast.org/clinical_breakpoints). Abbreviations: MIC, minimum inhibitory concentration; S, susceptible; R, resistant; CZA, ceftazidime/avibactam; IPM, imipenem; ETP, ertapenem; MEM, meropenem

In order to access the fitness cost of the *bla*$_{KPC-98}$-encoding plasmid, serial culture of the bacterial isolate was performed for 240 hours without the selective pressure of an antibiotic. Three randomly picked colonies were selected every 12 hours for PCR screening of the carbapenemase gene. The final PCR amplifications were vizualized through gel electrophoresis (**Figure 20**). After 216h hours (approximately 180 generations), some colonies of the FMUL503 isolate start losing the resistance gene, while all colonies of the control strain remain positive for the *bla*$_{KPC}$ gene until the end of the assay.

Additionally, one random colony of the last serial culture (240h; approximately 200 generations) was selected for disk diffusion test and exhibited susceptibility against CZA due to the loss of the *bla*$_{KPC-98}$ gene.

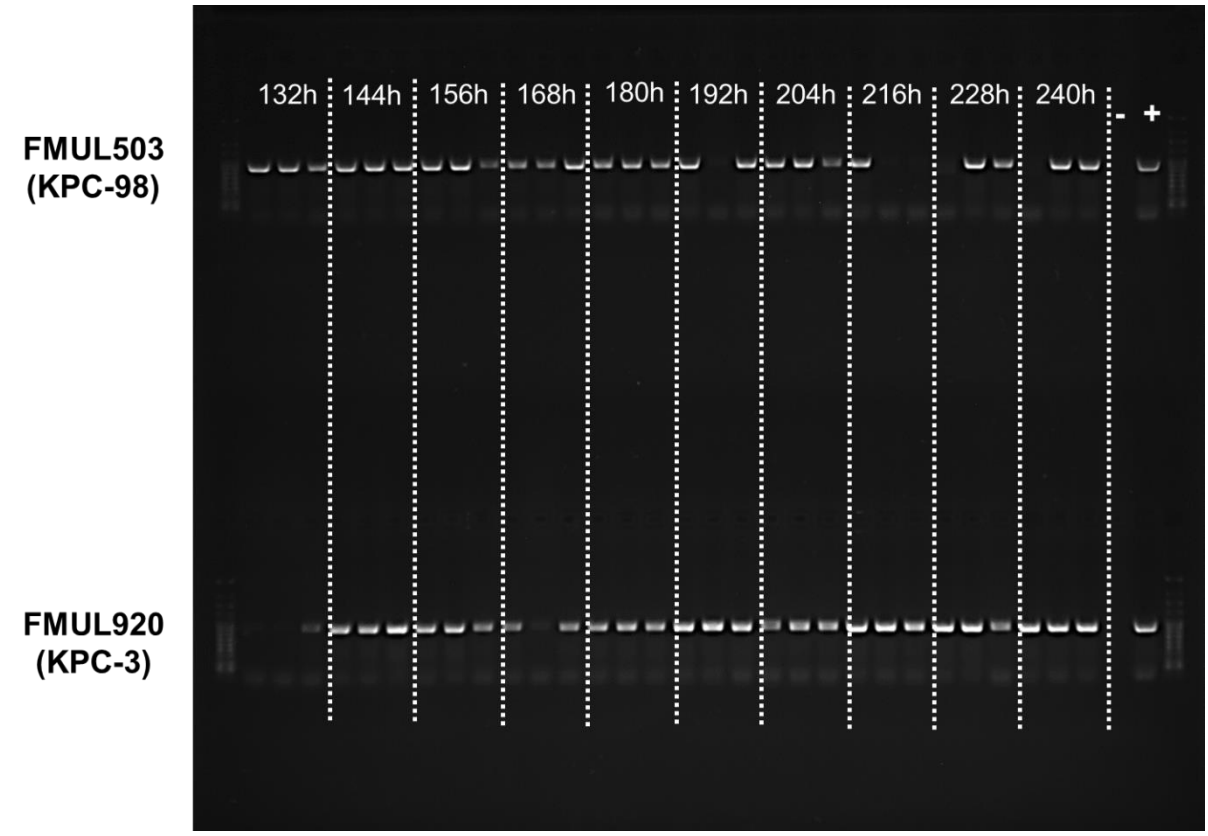

Figure 20 - Visualization, through gel electrophoresis, of the presence of the *bla*$_{KPC}$ gene in FMUL503 (above) and FMUL920 (below) during the last 5 days of the plasmid stability protocol. The dashed lines represent a 12 hours / 10 generation gap between the sets of 3 colonies tested for the presence of the carbapenemase gene through PCR amplification. Molecular weight marker: NZYDNA Ladder VIII. -, negative control; +, positive control.

4. Discussion

The antibiogram tests revealed a high resistant profile for the *Klebsiella* strains characterized in this study. The carbapenem susceptibility rates were inferior to 10%, which is worrisome, since carbapenems are considered last resort antibiotics against *Enterobacterales* infections [104]. Ceftazidime-avibactam was recently introduced in the pharmaceutical market as a new treatment option for the serious and difficult-to-treat infections,[105] and even though the spreading of this novel antibiotic did not reach worldwide proportions, several resistant strains against ceftazidime-avibactam have been found during this study.

Among the *Klebsiella* strains found in HAIs in Portugal, *K. pneumoniae* is undoubtedly the most commonly found species. Nevertheless, other members of this genus, frequently misidentified in clinical practice, can also harbour the same resistance genes and become a serious healthcare since it is not as studied as *K. pneumoniae*. The identification of these species through PCR amplification and sequencing of their 16S rDNA remains a reliable tool, as it showed great concordance with the WGS data.

Previous *K. pneumoniae*-focused studies in Portugal had demonstrated a different molecular epidemiology when compared to most countries in Europe - CG258 dominated [106] The predominance of clones such as ST13 and ST307 in this study confirms the unique molecular profile of *K. pneumoniae* in Portuguese health facilities, which origin remains unknown although emigration fluxes may play a role in the establishment of these high-risk clones. Within the national healthcare centres included in this study, evident differences could be found when looking into the most predominant lineages. Hospital 1, a tertiary care university hospital centre located in Lisbon, exhibited a large diversity of lineages being ST17-KL25-O5, ST147-KL64-O2, two ST13 subpopulations, and ST307-KL102-O2 the most represented ones. Hospital 2, also located in Lisbon, also had ST17-KL25/O5 and ST13-KL3-O1 as the most predominant *K. pneumoniae* lineages. The northern healthcare facility, Hospital 3, presented a high number of ST147-KL64-O2 *K. pneumoniae* strains. While both ST17-KL25-O5 and ST147-KL64-O2 lineages has been recently identified as a globally disseminated subpopulation which started to emerge since the mid-80s [107,108], the ST13 lineages were only registered in Portuguese hospitals [106], and the ST307-KL102-O2 in a 2019 oubreak in north-eastern Germany [109]. An outbreak of ST11-KL105-O2 strains was also detected during the SARS-CoV-2 pandemic in Hospital 3. The ST11 is widely spread across the globe and this particular lineage has been recently reported in Portuguese and Spanish hospitals [110,111].

Other members of the *Kp*SC were also detected in this study, namely *K. quasipneumoniae* and *K. variicola*. From these, two *K. quasipneumoniae* strains shared the same lineage (ST414-KL123-O3), which was also detected 1-year prospective cohort study in a US healthcare facility [112]. The same study also reported some *K. variicola* strains though none of them shared the same epidemiological markers as the strains detected in Portuguese hospitals, thus pinpointing the lack of studies regarding these species in HAIs.

Most of the *K. aerogenes* strains in this study belonged to the ST93-O2, a dominant global clone associated with *K. aerogenes* infections, being previously reported in the United States, in multiple

multidrug-resistant outbreaks in France [113] and most recently, in carbapenemase-producing strains in Brazil.[114].

The ST2-KL74 lineage was predominant among the *K. oxytoca* strains. Recent genomic studies have indicated ST2-KL74 as one of the most dominant clones across clinical strains, with a high number of cases reported in the United States and European countries [115]. Little is known about the characteristics of *K. oxytoca* capsules, but previous reports have identified *K. pneumoniae*-associated in *K. oxytoca* strains [116] The fact that KL74 was predominant among the *K. oxytoca* strains collected demonstrate the ability of this species to exchange DNA with *K. pneumoniae* and, thus, representing a potential reservoir of resistance and virulence genes. Among the *K. michiganensis* found in this study, one ST88 clone was detected. This clone, although not often reported, was already described in three different continents [117]. Furthermore, *K. ornithinolytica* and *K. pasteurii* strains were found in this study but due to sequencing problems and/or the lack of appropriate molecular typing scheme, it was not possible to properly identify the lineage of these strains.

A strong correlation could be found between the lineages and carbapenemase produced in *K. pneumoniae*. For instance, the ST17-KL25-O5 lineage were frequently found *bla*$_{\text{OXA-181}}$ carbapenemase. The occurrence an *K. pneumoniae* outbreaks sharing these exact characteristics has been reported in Africa [118,119], thus tracing a possible origin of this bacterial population. The *bla*$_{\text{KPC-3}}$ gene was strongly associated with the ST13-KL3-O1, while four ST13-KL19-O1 *K. pneumoniae* strains were found co-producing *bla*$_{\text{KPC-3}}$ and *bla*$_{\text{OXA-181}}$. This is evidence of an ongoing international dissemination of a KPC-3/ST13-producing clone from Portugal [120]. The ST147-KL64-O2 lineage was also associated with *bla*$_{\text{OXA-181}}$/*bla*$_{\text{KPC-3}}$ production. This lineage is highly prevalent carbapenem-resistant clone in Asian and European countries but in association with the *bla*$_{\text{NDM-1}}$, *bla*$_{\text{KPC-3}}$, or *bla*$_{\text{OXA-48}}$ [108], which indicates that this lineage as great affinity to a large variety of carbapenemase-encoding MGE. The NDM-1-producing ST11-KL105-O2 *K. pneumoniae* have already been previously studied by this research group, in which we suggested the Asian region, particularly China, that has been affected with several outbreaks ST11 clones expressing NDM-1 and OXA-48-like carbapenemases, as the possible origin of this outbreak [103]. More recently, the same NDM-1-producing lineage has been reported in Spain [111], which indicates initial dissemination of this high-risk clone in Europe. Surprisingly only two of the ST307-KL102-O2 strains harboured the *bla*$_{\text{KPC-3}}$, despite previous reports of strong association between this carbapenemase gene and ST307 clones [121]. The *bla*$_{\text{GES-5}}$ gene and *bla*$_{\text{IMP-4}}$ were also identified in clinical strains of *K. pneumoniae*, whith *bla*$_{\text{IMP-4}}$ never been previously reported in a Portuguese health facility.

Co-production of KPC-3 and OXA-181 was confirmed in 6 *K. pneumoniae* strains, 5 of them belonging to the ST13-KL19-O1 lineage. These strains presented resistance to five different antibiotic classes, but susceptibility against ceftazidime-avibactam. The *bla*$_{\text{KPC-40}}$ and *bla*$_{\text{KPC-70}}$ carbapenemase genes, recently discovered in European countries and associated with reduced susceptibility against ceftazidime-avibactam [122,123], were also found in the clinical ST13-KL3-O1 strains in this study. One novel KPC variant, denominated as KPC-98, was identified in a ceftazidime-avibactam resistant strain of the ST13-KL19-O1 lineage. Undoubtedly, ST13 clones are the main carriers of resistance determinants among HAIs in Portuguese hospitals and further awareness must be given to monitor and decrease the spread of this high-risk clone in clinical settings.

The *bla*$_{KPC-3}$ gene was also identified in most *K. quasipneumoniae* and *K. variicola* strains. The few reports KPC-3 production in these species [72,77], indicate that these strains are frequently misidentified as K. pneumoniae and, due to a lack of studies on these *Kp*SC members, no widespread carbapenem-resistant clones or lineages were described to our best knowledge.

The *bla*$_{OXA-181}$ carbapenemase gene was predominant in the *K. aerogenes* strains extracted from clinical cases, similar to what was recently observed in Lebanon.[124]. Only one isolate harboured the *bla*$_{KPC-3}$ gene, even though the presence of *bla*$_{KPC}$ in carbapenem-resistant strains appears to be more frequent than *bla*$_{OXA-181}$ [114,125]. This study also presents the first report of two *K. aerogenes* strains harbouring the *bla*$_{GES-5}$ and the *bla*$_{GES-5}$ + *bla*$_{KPC-3}$ genes. Previous studies have demonstrated the ability of *K. aerogenes* to acquire resistant determinants commonly found in *K. pneumoniae* [126], thus the necessity of studying the genetic environment of these carbapenemase genes in order to establish a possible relationship with resistance plasmids found in *K. pneumoniae* clinical strains.

K. oxytoca strains harbouring the *bla*$_{OXA-48}$ gene have been previously described in northern African countries,[127,128] however, those strains exhibited different lineages from those identified in Portuguese hospitals. Mobile genetic elements, such as transposons, may have been responsible for the spreading of *bla*$_{OXA-48}$ gene in *K. oxytoca* strains in Portugal. Furthermore, this is the first report of KPC-3 and KPC-3 + OXA-181 production in *K. michiganensis* strains. Since *K. michiganensis* is easily misidentified as *K. oxytoca*, the first clinical case of KPC-3–producing *K. michiganensis* in Europe was only reported in 2019 [129]. Little is known about the genetic characteristics of this species and associated mobile genetic elements. We also report for the first time *K. pasteurii* and *K. ornithinolytica* harbouring the *bla*$_{KPC-3}$ gene, even though a MDR *K. ornithinolytica*, misidentified as *K. oxytoca*, carrying the *bla*$_{OXA-48}$ gene was described in Mexico.

The acquisition of carbapenemase genes by plasmids from different incompatibility groups favours a more efficient dissemination between the same or different species, meaning it's not uncommon to find the same carbapenemase gene linked to several plasmid families [130]. If by one hand, there's a clear difference between the carbapenemases produced by *Kp*SC, *K. aerogenes*, and *Ko*SC strains, the same is not observed for the plasmid types found in these same strains. In all three groups, Col-like and IncF types represented over 70% of all the plasmid families found. The *K. aerogenes* strains presented less diversity of Inc-types, a clear bigger ratio of IncX3 and and Col-like, and smaller number of IncFIA plasmids when comparing with other groups.

Col-like plasmids have been increasingly reported as versatile AMR gene capture platforms among the Enterobacteriaceae family [131], with even reports of harbouring *bla*$_{KPC-2}$ in NTE$_{KPC}$-IId [42], even though that is not the case as it will be further demonstrated.

Interestingly, *bla*$_{KPC-3}$-harbouring IncFIA plasmids have been previously reported in CG258 *K. pneumoniae* strains in the United States [132] and then later in ST147 clinical strains in a Portuguese Hospital [133], which suggests a link to some of the *bla*$_{KPC-3}$-harbouring plasmids present in this study. For *K. oxytoca* and *K. aerogenes*, only a report of IncFIA plasmids harbouring *bla*$_{NDM}$-like genes was found [132], which diverges from the resistome described in the strains found in this study.

The IncFIB and IncFII groups represent over 40% of all plasmid families found in the analysed strains. In *K. pneumoniae*, both IncFIB and IncFII plasmids were widely identified carrying a great variety

of carbapenemase genes [134–138]. IncFIB plasmids were also responsible for the dissemination of *bla*$_{KPC-2/3}$ in clinical strains of *Ko*SC in Spain [84]. Associated with the *bla*$_{KPC-2}$, it can be found IncFII plasmids in *K. aerogenes* [139] and *K. oxytoca* [140]. Moreover, it is common to found multiple replicon IncFIB/IncFIB plasmids harbouring carbapenemase genes in *K. pneumoniae* [141,142], *K. variicola* [76], *K. aerogenes* [124], and *K. michiganensis* [143]. Portuguese healthcare facilities are no exception as this Inc-types were already described harbouring the *bla*$_{KPC-3}$ gene [144].

The presence of IncX3 plasmids in a significant scale in all species groups is also worth examination, as a massive occurrence of *K. pneumoniae* strains carrying the *bla*$_{OXA-181}$ gene located onto an IncX3 plasmid were previously described in Portugal and Portuguese-speaking African countries [144].

When trying to reconstruct the *bla*$_{KPC}$-harbouring plasmids of some of the *Klebsiella* strains of this study through the BRIG tool, it was possible to notice a great level of resemblance between plasmids of different strains. In fact, five different species (*K. pneumoniae, K. quasipneumoniae, K. variicola, K. aerogenes*, and *K. oxytoca*) of seven different lineages retrieved from two healthcare facilities presented this large plasmid, including not only KPC-3 producers, but also the CZA-resistant KPC-40, KPC-70, and KPC-98-producing strains. The plasmid providing the backbone for this reconstruction, pNY9_2, is a 140 kb IncFIA/IncFII plasmid that encodes multiple resistance determinant, such as *bla*$_{KPC-3}$, *bla*$_{OXA-9}$, and *bla*$_{TEM-1A}$ in a United States hospital [145]. This plasmid should not be discarded as possible origin since its Inc-types match the ones frequently found in the *Klebsiella* strains. TEM-1 carbapenemase was present in all plasmids but one (KPC-40-producing FMUL672 *K. pneumoniae* isolate), while OXA-9 β-lactamase was observed in only half of these plasmids. However, the presence of *bla*$_{OXA-9}$ and other resistance genes is part of an independent event caused by what appears to be a resistance gen cassette inserted in a MGE such as a composite transposon.

Only one KPC-3 producing isolate (a ST147-KL64-O2 *K. pneumoniae*) appears to carry a smaller and distinct plasmid that has the *bla*$_{KPC-3}$ gene as the only resistant determinant. The backbone plasmid, pWI1-KPC3 was extracted from a clinical *E. coli* strain from France. The fact that this KPC-3 producing isolate was found in the northern region hospital is a clear evidence that carbapenemase-encoding MGE are not evenly distributed across the Portuguese healthcare facilities.

Nonetheless, all the bla$_{KPC}$-like genes were found within the Tn*4401*d transposon as this is the main vehicle of dissemination of KPC dissemination across Portuguese hospitals. The fact that *bla*$_{KPC-40}$, *bla*$_{KPC-70}$, and *bla*$_{KPC-98}$ share the same MGE as *bla*$_{KPC-3}$, is indicative that these variants appeared in Portuguese HAIs due to a mutagenic event and not due to HGT of an already circulating KPC-variants. As previously established, the main Tn*4401* isoforms exhibit differences in small nucleotide deletions between the ISKpn7 transposase and the bla$_{KPC}$ gene, and that those deletions affect positively the KPC expression when comparing with the original Tn*4401*b isoform [37]. The Tn*4401*b is the most commonly identified isoform in clinical strains worldwide, however, Tn*4401*d, ever since its first description in 2012 [146], has been identified in *K. pneumoniae* and *E. coli* strains harbouring IncF or IncX3 plasmids from United States healthcare facilities [132,147]. This isoform also confirmed the ability to transpose from the plasmid to the bacterial chromosome, contributing to an increase resistance against CZA even without

the selective pressure of this antibiotic [147]. That phenomenon can only be confirmed in the strains of this study through long-read sequencing.

All the *bla*$_{OXA-181}$-harbouring plasmids shared great similarities with the 50kb p010_B-OXA181 plasmid, despite being found in three different species: *K. pneumoniae*, *K. aerogenes*, and *K. oxytoca*. This IncX3 plasmid was retrieved from a river sample in Switzerland [148], which establish a clear connection between clinical and environmental contexts through the dissemination of carbapenemase-encoding MGE. It has been established that the *bla*$_{OXA-181}$ gene can be found in different genetic environments depending on the type of plasmid that is inserted. For instance, when found in the bacterial chromosome, or in IncT, ColKP3 plasmids, the *bla*$_{OXA-181}$ gene is often found within the Tn*2013* transposon [49]. In IncX3 plasmids, the elements of the Tn*2013* are truncated and it is possible to detect the ISKpn19 and *qnrS1* resistance gene are downstream of *bla*$_{OXA-181}$ and the IS3000 is upstream of this carbapenemase gene; all these elements are flanked by a composite transposon consisting of two copies of the insertion sequence IS26 [149]. These elements were detected in all OXA-181-producing *Klebsiella* strains selected for plasmid reconstructed, apart from IS3000, which was not detected by the MobileElementFinder software, and the IS26 flanking regions, which only share a 70% match with all the plasmids reconstructed in this study. The confirmation and proper identification of the IS element flanking the gene of interest can only be achieved through long-read sequencing, nevertheless these results might indicate the presence of a novel composite transposon variant harbouring the *bla*$_{OXA-181}$ gene.

In a ST2-KL74-O3 *K. oxytoca* isolate, *bla*$_{OXA-48}$ harbouring plasmid was detected. Unsurprisingly the plasmid and MGE found in association with this gene were very different from the ones observed for *bla*$_{OXA-181}$, sharing an almost perfect match with the p2018C08-141-66k plasmid. In this particular case, the *bla*$_{OXA-48}$ gene was the single resistant determinant found in the plasmid and very few MGE were found in this plasmid. Similar to what can be observed for Tn*4401* and *bla*$_{KPC}$, the *bla*$_{OXA-48}$ gene is frequently associated with the Tn*1999* tranposon, whose variants have been well characterized over the past years [49]. However, the IS1999 was not found neither upstream or downstream the carbapenemase gene. The gene appears to be surrounded by IS1 insertion sequences, which leads to two main hypothesis: *bla*$_{OXA-48}$ is either associated with Tn*1999.3*, a variant first described within an *E. coli* strain in Italy where the carbapenemase gene is flanked by two copies of IS1R [150], or with Tn*6237*. The Tn*6237* is a large 20kb IS1R-based composite transposon which contains an inverted ΔTn*1999.2* [49]. It has been described into different chromosomal insertion sites and several plasmid types among *E. coli* and *K. pneumoniae* [151]. The ability of this MGE to integrate in the bacterial genome and subsequent clonal dissemination was pointed as one of the biggest factors for the increase of OXA-48-producing *E. coli* in the United Kingdom [152]. At last, the possibility of the presence of a novel transposon, variant of Tn*1999* or Tn*6237*, should not be discarded since little is described on the genetic environment of OXA-48-producing *K. oxytoca* strains.

During the SARS-CoV-2 pandemic, an outbreak of NDM-1-producing *K. pneumoniae* ST11-KL105-O2 was reported in Hospital 3. Due to the clear similarity between the strains in this outbreak, the dissemination of the NDM-1 carbapenemase was instigated by vertical dissemination, rather then HGT as we previously approached [103]. Due to the limitations of short-read sequencing, it was unable to

properly reconstruct of the *bla*$_{NDM-1}$ encoding plasmid present in this outbreak. Although it is difficult to determine the plasmid size and which exact MGE may be in association with the *bla*$_{NDM-1}$ gene, elements of the Tn*125* were found, particularly *ble*$_{MBL}$, a bleomycin resistance gene next to the *bla*$_{NDM-1}$ and two insertion sequences, that share 70% resemblance with IS26. It must be also take into account that although Tn*125* played a major role in early dissemination of the *bla*$_{NDM-1}$ gene, this MGE have been slowly overtaken by other MGE such as IS26-flanked pseudo-composite transposons [64]. Only by accessing the complete sequence of the plasmid, it can be determined if this metallo-β-lactamase is encoded within a Tn*125* or a novel IS26-variant composite transposon.

One ST93-KL68-O5 *K. aerogenes* isolate presented co-production of KPC-3 and GES-5 in different plasmids. The *bla*$_{GES-5}$ gene was found in a small plasmid with little over 10kb (**Figure 18**), whose backbone plasmid pKP-M1144 was described in a clinical *K. pneumoniae* isolate from Spain. Upstream the Tn*5403* transposon the *bla*$_{GES-5}$ gene can be detected in addition with two other resistance genes (*bla*$_{BEL-1}$, and *aac(6')-Ib*). This carbapenemase gene is frequently found in association with class 1 integrons [65], however this element was not found in this plasmid since part of the plasmid sequence next to the *bla*$_{GES-5}$ is missing, thus determine with precision the MGE responsible for the mobilization and dissemination of this gene is currently not possible. In Portugal, the *bla*$_{GES-5}$ gene was described in a plasmid that share great similarity with the plasmid described in this study's bacterial isolate [153]. This small 12kb ColE1 plasmid carries the class 3 integron, In1144, in association with a gene resistance cassette harbouring the *bla*$_{IMP-8}$, *bla*$_{GES-5}$, *bla*$_{BEL-1}$, and *aacA4* genes. The complete sequence of the *K. oxytoca* plasmid will allow to determine if this In1144 class 3 integron is also present and, if so, it is evidence of an interesting case of evolution of resistance determinants in Gram-negative bacteria.

This is the first national report of the metallo-β-lactamase IMP-4 in a ST20-KL28-O1 *K. pneumoniae* plasmid. Due to short-read sequencing limitations, it was not possible to uncover the sequence and length of this *bla*$_{IMP-4}$-encoding plasmid. This carbapenemase gene was found in a resistant gene cassette with other drug resistance determinants (*sul1*, *catB3*, and *aac(6')-Ib3*) which is characteristic of class 1 integrons. The report of class 1 integrons harbouring this set of resistance genes can be found in several clinical *Klebsiella* strains from Asian countries [154,155].

Among the strains this study, it can be highlighted a novel KPC-3 variant, KPC-98, in a clinical ST13-KL19-O1 *K. pneumoniae* isolate. This isolate presented MDR, including to all carbapenems tested, and to CZA, later confirmed by ETEST, and susceptibility against ciprofloxacin, cefoxitin, and tigecycline. The KPC-98 protein sequence presented one amino acid substitution (R164H) located in the KPC Ω-loop region (164-179) compared with KPC-3. Genetic environment analysis revealed that the *bla*$_{KPC-98}$ is located within the transposon Tn*4401*d and in the same plasmids that carry the *bla*$_{KPC-3}$ gene in *Klebsiella* strains extracted from HAIs in Portugal.

In order to evaluate if the KPC-98 enzyme is directly responsible for CZA resistance, its gene was amplified in a plasmid vector and introduced in chemically competent *E. coli* cells. These recombinant clones were further tested for antibiotic resistance by disk diffusion test and ETEST, exhibiting acquired resistance to CZA due to the presence of KPC-98, while remaining susceptible to carbapenems. Nevertheless, these findings corroborate with what has been previously found for other KPC-3 variants,

since carbapenem resistance is determined by other factors such as gene copy number, efflux pumps, and porin mutation [156].

The fitness cost of the *bla*$_{KPC-98}$-encoding plasmid was assessed through serial culture of the bacterial isolate for 240 hours without the selective pressure of an antibiotic. After 216h hours (9 days), which corresponds to approximately 180 bacterial generations, some colonies of the *bla*$_{KPC-98}$-encoding isolate start losing the resistance gene, while all colonies of the control strain remain positive for the *bla*$_{KPC-3}$ gene until the end of the assay. In the end of the assay (240h; approximately 200 generations), disk diffusion test was performed for one random FMUL503 colony and exhibited susceptibility against CZA due to the loss of the *bla*$_{KPC-98}$ gene. This is evidence of a higher fitness cost of KPC-98 for the bacteria when compared to the commonly identified in clinical strains, KPC-3. The high fitness cost of this carbapenemase may be an explanation for why this carbapenemase was not described in any study since it first appeared in a Portuguese *K. pneumoniae* clinical isolate. While the main worldwide KPC carbapenemases (KPC-2/-3) are well characterized in terms of fitness cost for the cell [157,158], the same can not be observed for novel KPC variants that confer resistance of different class of enzymes. However, a recent study reveals that a novel KPC variant associated with imipenem/relebactam resistance, one of the most recent antibiotic combinations introduced in the market, represented a high fitness cost for *K. pneumoniae* [158]. This goes in line with the latest findings of this work, suggesting that a high fitness cost is directly correlated with a high level of drug resistance. In parallel, the successful evolution of resistance plasmids is dependent on the enhancement of associated fitness costs, and when exposed to a large range of antibiotics, those resistance plasmids may proliferate more efficiently.

5. Conclusion

This master's dissertation focused on the study of healthcare associated *Klebsiella* species infections in Portugal. From this work, several key findings and important implications emerged. The prevalence of MDR among the clinical strains is concerning, with extremely low susceptibility rates to last-resource antibiotics, namely carbapenems, emphasizing the pressing need for effective infection control strategies.

The predominant species identified was *K. pneumoniae* and the distribution of its lineages was not uniform across hospitals, indicating the importance of local epidemiological surveillance. Other members of the *Klebsiella* genus, frequently misidentified in clinical practice, were also found encoding resistance determinants. Only through accurate species-level identification it is possible to understand the full extent of antibiotic resistance dissemination within non-*K. pneumoniae* species in healthcare settings. Molecular methods should be employed to complement conventional identification techniques.

Carbapenem resistance was primarily mediated by KPC-3 and OXA-181. This study also included an NDM-1-producing *K. pneumoniae* outbreak, a novel resistance determinant (KPC-98), and co-production of two types of carbapenemases observed in several strains, showing the degree of complexity of resistance mechanisms within this genus.

MGEs, such as plasmids and transposons, played a vital role in the dissemination of carbapenemase genes among different *Klebsiella* strains. The main evidence is the predominance of the same replicon types (Col- and IncF- types) and the presence of common plasmids/transposons across multiple species and hospitals. As the novel antibiotics development pipeline is too slow when compared with the increasing rate of antimicrobial resistance, the research herein accomplished points that the prevention of HGT mechanisms and detection of MGE for therapeutic/diagnosis purposes present remarkable potential in the battle against the antibiotic resistance crisis.

This work also reports a novel KPC variant – KPC-98 – that is capable to confer resistance against novel antibiotic combination ceftazidime-avibactam, while increasing the fitness cost of its bacterial host. Understanding these novel carbapenemase variants is needed to characterize their clinical significance, transmission dynamics, and fitness costs associated. Only with all this information, it possible to develop targeted therapies and evaluating the potential for resistance spread.

Considering all these findings, some recommendations can be defined: enhance surveillance measures in order to monitor the prevalence and distribution of carbapenem-resistant *Klebsiella* species within healthcare facilities, implement strict infection control measures to prevent the spread of these pathogens, promote collaboration between countries in addressing the global challenge of antibiotic resistance (sharing data, experiences, and best practices), and introduce antibiotic stewardship programs that promote responsible antibiotic use, reducing the selective pressure that drives antibiotic resistance, thus preserving the effectiveness of last-resort antibiotics.

Some limitations on this study should also be taking into account, since short-read sequence data is not able to provide full plasmid sequences, or copies of MGE encoding resistance genes in the genome. However, this is, to the date, the first in-depth genomic national multi study of carbapenem resistance *Klebsiella* spp. clinical strains with particular focus on the mobilome and good representation

of non-*K. pneumoniae* species, in which there is a great lack of global scientific evidence. Future research should investigate the environmental reservoirs of resistance genes, such as water sources and hospital environment with the aim of outlining strategies to reduce the environmental spread of resistance. Furthermore, hybrid assembly with long-reads and short-read of the *Klebsiella* strains of interest will be applied in order to fill the gaps of this research and provide insights on the gene exchanges between the mobilome and resistome of these resistance strains.

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7. Annexes

Table S1 – List of 148 complete sequenced carbapenemase-encoding *Klebsiella* plasmids. Retrieved from the Genbank database on October 27th 2022.

Accession	ResFinder	PlasmidFinder	Plasmid	Species	Country	Year
MN370927.1	*bla*NDM-1		pNDM1-M11	K. *pneumoniae*	Thailand	2020
MN370928.1	*bla*NDM-1		pNDM1-M33	K. *pneumoniae*	Thailand	2020
MN370929.1	*bla*NDM-1		pNDM1-M118	K. *pneumoniae*	Thailand	2020
MN816229.1	*bla*NDM-1		pNDM1_Isoform1	K. *pneumoniae*	Peru	2020
MN816230.1	*bla*NDM-1		pNDM1_Isoform2	K. *pneumoniae*	Peru	2020
MN816231.1	*bla*NDM-1		pNDM1_Isoform3	K. *pneumoniae*	Peru	2020
MN816232.1	*bla*NDM-1		pNDM1_Isoform4	K. *pneumoniae*	Peru	2020
MN816233.1	*bla*NDM-1		pNDM1_Isoform5	K. *pneumoniae*	Peru	2020
KY215945.1	*bla*OXA-519		pOXA-519	K. *pneumoniae*	Belgium	2020
CP026019.1	*bla*VIM-1		p13190-1	K. *pneumoniae*	China	2013
MN371150.1	*bla*OXA-232	ColKP3	pC105-OXA232-ColE	K. *pneumoniae*	India	2019
CP095471.1	*bla*KPC-3	ColKP3; IncFIA; IncFIB; IncR	Kpn-WC17-014-03	K. *pneumoniae*	USA	2017
CP090627.1	*bla*KPC-3	ColKP3; IncFIB	unnamed1	K. *pneumoniae*	Italy	2021
CP053774.1	*bla*OXA-232	ColKP3; IncFIB; IncHI1B	pColKP3_1	K. *pneumoniae*	India	2019
CP090472.1	*bla*OXA-232	ColKP3; IncFIB; IncR	p4	K. *pneumoniae*	China	2020
CP037744.1	*bla*KPC-2	ColKP3; IncFIB; IncR	pDHQP1701672_amr	K. *pneumoniae*	USA	2016
MN310375.1	*bla*NDM-1	IncC	pQD1501-Ct1	K. *quasipneumoniae*	China	2015
CP027039.1	*bla*OXA-48	IncC; IncFIB; IncX	IncLM	K. *pneumoniae*	Greece	2013
ON023484.1	*bla*NDM-5	IncC; IncX	KP113-NDM-5	K. *pneumoniae*	China	2020
CP065475.1	*bla*IMP-4; *bla*NDM-1	IncFIA; IncFIB; IncR	pKOX7525_1	K. *michiganensis*	China	2020
CP070565.1	*bla*KPC-2	IncFIA; IncFIB; IncR	p51248_KPC	K. *pneumoniae*	Czechia	2019
CP070599.1	*bla*KPC-2	IncFIA; IncFIB; IncR	pA9853_KPC	K. *pneumoniae*	Czechia	2019
CP083022.1	*bla*OXA-48	IncFIA; IncFIB; IncR	p1-6611.48-OXA48	K. *pneumoniae*	Switzerland	2017
CP083027.1	*bla*OXA-48	IncFIA; IncFIB; IncR	p1-6604.68-OXA48	K. *pneumoniae*	Switzerland	2017
CP083017.1	*bla*OXA-48	IncFIA; IncFIB; IncR	p1-6709.15-I-OXA48	K. *pneumoniae*	Switzerland	2018
CP067905.1	*bla*KPC-2	IncFIA; IncFIB; IncR	pC250_3	K. *pneumoniae*	USA	2016
CP067545.1	*bla*KPC-2	IncFIA; IncFIB; IncR	pC687_2	K. *pneumoniae*	USA	2017
CP070560.1	*bla*KPC-3	IncFIA; IncFIB; IncR	p51069_KPC	K. *pneumoniae*	Czechia	2019
CP092761.1	*bla*KPC-3	IncFIA; IncFIB; IncR	unnamed2	K. *pneumoniae*	Italy	2020
CP058327.1	*bla*KPC-40	IncFIA; IncFIB; IncR	pIT-1825-FIIK1	K. *pneumoniae*	Italy	2018
CP097413.1	*bla*OXA-505; *bla*OXA-48	IncFIA; IncFIB; IncR	unnamed2	K. *pneumoniae*	Spain	2016
CP067755.1	*bla*KPC-2	IncFIA; IncFIB; IncR	pC320_3	K. *pneumoniae*	USA	2016
CP067901.1	*bla*KPC-2	IncFIA; IncFIB; IncR	pC251_2	K. *pneumoniae*	USA	2016
CP067576.1	*bla*KPC-2	IncFIA; IncFIB; IncR	pC608_1	K. *pneumoniae*	USA	2017
CP083008.1	*bla*KPC-2	IncFIA; IncFIB; IncR	p1-6711.43-KPC2	K. *pneumoniae*	Switzerland	2018
CP083001.1	*bla*KPC-3	IncFIA; IncFIB; IncR	p1-6712.08-KPC3	K. *pneumoniae*	Switzerland	2018
CP070525.1	*bla*KPC-2	IncFIA; IncFIB; IncR	p47693_KPC	K. *pneumoniae*	Czechia	2018

CP068017.1	*bla*OXA-48	IncFIA; IncFIB; IncR	pMS3802OXARMA	K. *pneumoniae*	Spain	2016
CP067595.1	*bla*KPC-2	IncFIA; IncFIB; IncR; IncX	pC601_3	K. *pneumoniae*	USA	2017
CP067700.1	*bla*KPC-2	IncFIA; IncFIB; IncR; IncX	pC4688_2	K. *pneumoniae*	USA	2016
CP033628.1	*bla*KPC-2	IncFIA; IncR	unnamed3	K. *pneumoniae*	Italy	2013
CP026369.1	*bla*IMP-4	IncFIB	pA708-1	K. *quasipneumoniae*	China	2014
CP098358.1	*bla*KPC-2	IncFIB	p13001-QIL	K. *pneumoniae*	USA	2003
CP022693.2	*bla*KPC-2	IncFIB	pAUSMDU00008079_02	K. *pneumoniae*	Australia	2012
CP056026.1	*bla*KPC-2	IncFIB	pKPN39428.2	K. *pneumoniae*	USA	2013
CP067573.1	*bla*KPC-2	IncFIB	pC678_1	K. *pneumoniae*	USA	2017
CP049601.1	*bla*KPC-2	IncFIB	pSECR18-2341_KPC	K. *aerogenes*	South Korea	2018
CP103607.1	*bla*KPC-2	IncFIB	pMB7231_2	K. *pneumoniae*	USA	2018
CP103551.1	*bla*KPC-2	IncFIB	pMB8806_1	K. *pneumoniae*	USA	2019
CP037930.1	*bla*KPC-3	IncFIB	p8788-IT	K. *pneumoniae*	Italy	2017
CP092754.1	*bla*KPC-3	IncFIB	unnamed1	K. *pneumoniae*	Italy	2020
CP092755.1	*bla*KPC-3	IncFIB	unnamed2	K. *pneumoniae*	Italy	2020
CP092760.1	*bla*KPC-3	IncFIB	unnamed1	K. *pneumoniae*	Italy	2020
CP092766.1	*bla*KPC-3	IncFIB	unnamed1	K. *pneumoniae*	Italy	2020
CP092767.1	*bla*KPC-3	IncFIB	unnamed2	K. *pneumoniae*	Italy	2020
CP061387.1	*bla*KPC-3	IncFIB	pCRE-213-2	K. *pneumoniae*	USA	2020
CP090624.1	*bla*KPC-3	IncFIB	unnamed1	K. *pneumoniae*	Italy	2021
CP090630.1	*bla*KPC-3	IncFIB	unnamed1	K. *pneumoniae*	Italy	2021
CP100309.1	*bla*KPC-31	IncFIB	pKpQIL_1021	K. *pneumoniae*	Italy	2021
CP100313.1	*bla*KPC-31	IncFIB	pKpQIL_1020	K. *pneumoniae*	Italy	2021
CP104374.1	*bla*NDM-1	IncFIB	unnamed1	K. *pneumoniae*	USA	2021
CP104662.1	*bla*NDM-1	IncFIB	unnamed2	K. *pneumoniae*	USA	2021
CP104680.1	*bla*NDM-1	IncFIB	unnamed2	K. *pneumoniae*	USA	2021
CP041642.1	*bla*NDM-4	IncFIB	pKP27-NDM4	K. *pneumoniae*	Vietnam	2015
CP095680.1	*bla*NDM-1	IncFIB; IncFII	p_kv_NDM1	K. *variicola*	Bangladesh	2016
CP095569.1	*bla*NDM-5	IncFIB; IncFII	p_dm336b_NDM5	K. *pneumoniae*	Bangladesh	2016
CP095619.1	*bla*NDM-5	IncFIB; IncFII	p_dm428b_NDM5	K. *pneumoniae*	Bangladesh	2017
CP095596.1	*bla*NDM-1	IncFIB; IncFII; IncR	p_dm129b_NDM1	K. *pneumoniae*	Bangladesh	2016
CP095582.1	*bla*NDM-5	IncFIB; IncFII; IncR	p_b217b_NDM5	K. *pneumoniae*	Bangladesh	2017
CP095782.1	*bla*KPC-3	IncFIB; IncFII; IncR	p120Kb	K. *pneumoniae*	Italy	2021
CP087148.1	*bla*KPC-41	IncFIB; IncFII; IncR	unnamed2	K. *pneumoniae*	China	2021
CP087153.1	*bla*KPC-41	IncFIB; IncFII; IncR	unnamed2	K. *pneumoniae*	China	2021
CP067553.1	*bla*KPC-2	IncFIB; IncFII; IncR	pC686_3	K. *pneumoniae*	USA	2017
CP034406.1	*bla*NDM-1; *bla*NDM-2	IncFIB; IncHI1B	pNH34.1	K. *pneumoniae*	Thailand	2014
CP034201.2	*bla*NDM-5	IncFIB; IncHI1B	pKpvST383L	K. *pneumoniae*	UK	2018
VCEE01000003.1	*bla*NDM-5	IncFIB; IncHI1B	pKpvST48_1	K. *pneumoniae*	UK	2018
CP091814.1	*bla*NDM-5	IncFIB; IncHI1B; IncR	pFQ61_ST383_NDM-5	K. *pneumoniae*	Qatar	2016
CP067814.1	*bla*KPC-2	IncFIB; IncHI1B; IncR	pC289_1	K. *pneumoniae*	USA	2016
VILG01000002.1	*bla*OXA-48	IncFIB; IncHI1B; IncR	pRYC-OXA48	K. *pneumoniae*	Spain	2012
CP067461.1	*bla*KPC-2	IncFIB; IncR	pC872_2	K. *pneumoniae*	USA	2016

CP067476.1	*bla*KPC-2	IncFIB; IncR	pC763_3	K. *pneumoniae*	USA	2016
CP067669.1	*bla*KPC-2	IncFIB; IncR	pC4695_1	K. *pneumoniae*	USA	2016
CP067681.1	*bla*KPC-2	IncFIB; IncR	pC4692_3	K. *pneumoniae*	USA	2016
CP067763.1	*bla*KPC-2	IncFIB; IncR	pC315_2	K. *pneumoniae*	USA	2016
CP067819.1	*bla*KPC-2	IncFIB; IncR	pC288_2	K. *pneumoniae*	USA	2016
CP067833.1	*bla*KPC-2	IncFIB; IncR	pC278_2	K. *pneumoniae*	USA	2016
CP067868.1	*bla*KPC-2	IncFIB; IncR	pC265_2	K. *pneumoniae*	USA	2016
CP067884.1	*bla*KPC-2	IncFIB; IncR	pC259_2	K. *pneumoniae*	USA	2016
CP067893.1	*bla*KPC-2	IncFIB; IncR	pC253_3	K. *pneumoniae*	USA	2016
CP067918.1	*bla*KPC-2	IncFIB; IncR	pC244_2	K. *pneumoniae*	USA	2016
CP067963.1	*bla*KPC-2	IncFIB; IncR	pC1016_2	K. *pneumoniae*	USA	2016
CP067969.1	*bla*KPC-2	IncFIB; IncR	pC237_1	K. *pneumoniae*	USA	2016
CP067443.1	*bla*KPC-2	IncFIB; IncR	pC962_1	K. *pneumoniae*	USA	2017
CP067527.1	*bla*KPC-2	IncFIB; IncR	pC699_2	K. *pneumoniae*	USA	2017
CP067539.1	*bla*KPC-2	IncFIB; IncR	pC691_3	K. *pneumoniae*	USA	2017
CP067614.1	*bla*KPC-2	IncFIB; IncR	pC593_2	K. *pneumoniae*	USA	2017
CP067624.1	*bla*KPC-2	IncFIB; IncR	pC591_2	K. *pneumoniae*	USA	2017
CP067634.1	*bla*KPC-2	IncFIB; IncR	pC588_3	K. *pneumoniae*	USA	2017
CP067639.1	*bla*KPC-2	IncFIB; IncR	pC587_2	K. *pneumoniae*	USA	2017
CP067719.1	*bla*KPC-2	IncFIB; IncR	pC344_1	K. *pneumoniae*	USA	2017
CP067744.1	*bla*KPC-2	IncFIB; IncR	pC335_1	K. *pneumoniae*	USA	2017
CP068609.1	*bla*KPC-2	IncFIB; IncR	p59493-R72.9	K. *pneumoniae*	USA	2018
CP067451.1	*bla*KPC-2	IncFIB; IncR	pC957_3	K. *pneumoniae*	USA	2016
CP095778.1	*bla*KPC-3	IncFIB; IncR	p112Kb	K. *pneumoniae*	Italy	2021
CP092904.1	*bla*NDM-1	IncFIB; IncR	pC_F11	K. *pneumoniae*	China	2019
CP068862.1	*bla*OXA-48	IncFIB; IncR	pRIVM_C017514_2	K. *pneumoniae*	Netherlands	2018
CP068871.1	*bla*OXA-48	IncFIB; IncR	pRIVM_C017077_3	K. *pneumoniae*	Netherlands	2018
CP068945.1	*bla*OXA-48	IncFIB; IncR	pRIVM_C019719_1	K. *pneumoniae*	Netherlands	2019
CP068951.1	*bla*OXA-48	IncFIB; IncR	pRIVM_C019672_2	K. *pneumoniae*	Netherlands	2019
CP068613.1	*bla*KPC-2	IncFIB; IncR	p45706-R73.3	K. *pneumoniae*	USA	2013
CP068617.1	*bla*KPC-2	IncFIB; IncR	p42362-R67.9	K. *pneumoniae*	USA	2013
CP067838.1	*bla*KPC-2	IncFIB; IncR	pC277_2	K. *pneumoniae*	USA	2016
CP055296.1	*bla*KPC-2	IncFIB; IncR	pKP55_2	K. *pneumoniae*	China	2020
CP068621.1	*bla*KPC-3	IncFIB; IncR	p41968-R65.8	K. *pneumoniae*	USA	2013
CP094890.1	*bla*KPC-3	IncFIB; IncR	Kpn-WC17-004-03	K. *pneumoniae*	USA	2017
CP094895.1	*bla*KPC-3	IncFIB; IncR	Kpn-WC17-005-03	K. *pneumoniae*	USA	2017
CP094904.1	*bla*KPC-3	IncFIB; IncR	Kpn-WC17-008-01	K. *pneumoniae*	USA	2017
CP061969.1	*bla*OXA-244; *bla*OXA-48	IncFIB; IncR	unnamed4	K. *pneumoniae*	China	2015
CP061974.1	*bla*OXA-48	IncFIB; IncR	unnamed4	K. *pneumoniae*	China	2018
CP067771.1	*bla*KPC-2	IncFIB; IncR; IncX	pC310_2	K. *pneumoniae*	USA	2016
CP067787.1	*bla*KPC-2	IncFIB; IncR; IncX	pC301_2	K. *pneumoniae*	USA	2016
CP067874.1	*bla*KPC-2	IncFIB; IncR; IncX	pC262_3	K. *pneumoniae*	USA	2016
CP067590.1	*bla*KPC-2	IncFIB; IncR; IncX	pC603_2	K. *pneumoniae*	USA	2017
CP067726.1	*bla*KPC-2	IncFIB; IncR; IncX	pC342_2	K. *pneumoniae*	USA	2017

CP067730.1	*bla*KPC-2	IncFIB; IncR; IncX	pC338_1	K. *pneumoniae*	USA	2017
CP067738.1	*bla*KPC-2	IncFIB; IncR; IncX	pC336_2	K. *pneumoniae*	USA	2017
CP067492.1	*bla*KPC-2	IncFIB; IncR; IncX	pC720_3	K. *pneumoniae*	USA	2017
CP067791.1	*bla*KPC-2	IncFIB; IncR; IncX	pC299_2	K. *pneumoniae*	USA	2016
CP067798.1	*bla*KPC-2	IncFIB; IncR; IncX	pC295_2	K. *pneumoniae*	USA	2016
CP067783.1	*bla*KPC-2	IncFIB; IncX	pC305_2	K. *pneumoniae*	USA	2016
ON261191.1	*bla*NDM-5	IncHI1B	pEGY22_CTX_15	K. *pneumoniae*	Egypt	2020
MK330869.1	*bla*KPC-2	IncQ1	pB29	K. *pneumoniae*	Brazil	2010
MK330868.1	*bla*KPC-2	IncQ1	pKPC05	K. *pneumoniae*	Brazil	2014
CP068028.1	*bla*IMP-4	IncR	pIMP-KP-13-9	K. *pneumoniae*	China	2013
OM791347.1	*bla*KPC-2	IncR	pD	K. *pneumoniae*	China	2022
OL744330.1	*bla*KPC-31	IncR	RFKPC	K. *pneumoniae*	Italy	2022
MT875328.1	*bla*KPC-41	IncR	pHS2953-KPC	K. *pneumoniae*	China	2021
OM791348.1	*bla*NDM-5	IncR	pE	K. *pneumoniae*	China	2022
MZ853143.1	*bla*KPC-2	IncX	pTJ30-KPC	K. *quasipneumoniae*	China	2015
MT028409.1	*bla*KPC-2	IncX	pBS407-3	K. *pneumoniae*	South Korea	2019
MF156708.1	*bla*KPC-2	IncX	p13294-KPC	K. *pneumoniae*	China	2020
MF156709.1	*bla*KPC-2	IncX	pE20-KPC	K. *aerogenes*	China	2020
MN064714.1	*bla*NDM-5	IncX	pY9645-NDM5	K. *pneumoniae*	China	2016
MZ363820.1	*bla*NDM-5	IncX	pKA-NDM-5	K. *aerogenes*	China	2020