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Phenotypic and molecular characterization of *Klebsiella pneumoniae* isolates of clinical origin resistant to ceftazidime/avibactam

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1 INTRODUCTION

1.1 Antimicrobial resistance: a global health threat problem

Antimicrobial resistance (AMR) has been recognized as one of the greatest global threats to the health of humans and animals, plants and ecosystems. In our globally connected world, AMR may rapidly spread anywhere. It renders ineffective the prevention and treatment of a growing range of infections caused by bacteria, parasites, viruses and fungi. Misuse and overuse of different antibacterial agents in the health care setting and in the agricultural industry are considered among the major reasons behind the emergence of AMR [1].

AMR takes place when bacteria, viruses, fungi and parasites change over time and no longer respond to drugs (i.e. antibiotics, antivirals, antifungals and antiparasitics), making infections harder to treat and increasing the risk of severe illness and death. Antimicrobial agents become ineffective, and infections persist in the body, increasing the risk of spread to other patients.

Despite different actions taken in recent decades to contain that problem, the trends of global AMR demonstrate no signs of slowing down.

1.2 Antibiotics: general characteristics and mechanisms of action

Antibiotics are essential drugs for the treatment of bacterial infections. These powerful medical tools have saved millions of lives since their discovery. However, their misuse

and the spread of antibiotic resistance are becoming an increasingly serious public health challenge.

Antibiotics are typically produced by naturally occurring microorganisms, in order to kill or inhibit the growth of other microorganisms residing in the same ecological niche. Additionally, chemical derivatives of natural antibiotics were also developed to improve pharmacokinetic\pharmacodynamic properties of the drugs and their activity spectrum. Conventionally, antibiotics target essential processes or structures of the bacterial cell, exerting a reversible (bacteriostatic) or irreversible (bactericidal) inhibitory effect, according to the drug's features, and can be classified according to their mechanisms of action. (*Figure 1)*

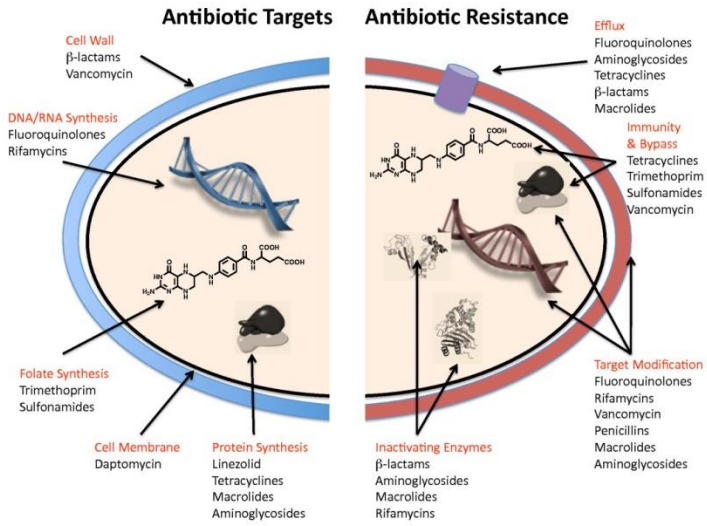

Figure 1. Overview of main antibiotic molecular targets and resistance mechanisms (adapted from Wright, G.D. Q&A: Antibiotic resistance: where does it come from and what can we do about it. BMC Biol ***8****, 123 (2010). https://doi.org/10.1186/1741-7007-8-123)*

1.2.1 Cell wall inhibition

Several antibiotics work by inhibiting the synthesis of the bacterial cell wall, which is essential for maintaining the shape and integrity of bacterial cells. By blocking its synthesis, antibiotics make bacteria vulnerable to their environment, leading to their death. This class include β-lactams (penicillins, cephalosporins, carbapenems, monobactams), glycopeptides, phosphomycin, polymyxins, cycloserine, polypeptides (bacitracin).

B-lactams antibiotics represent the largest, best known and most widely used group of antibiotics in clinical practice. They are divided into four different classes, using as a discriminating criterion the way in which the β-lactam ring binds to the rest of the molecule.

They are divided into:

- ✓ penicillins
- ✓ cephalosporins
- ✓ monobactams
- ✓ carbapenems

The first discovered antibiotic was penicillin, first isolated by Scottish scientist Alexander Fleming from mould (*Penicillium notatum*) samples that had contaminated a *Staphylococcus* culture dish, producing a clear ring (absence of bacterial growth) around the mould. He subsequently purified the active ingredient and discovered that it was capable of killing a wide range of harmful bacteria, including *Streptococcus*, *Neisseria meningitidis* and diphtheria bacillus. The use of penicillin as a therapeutic agent to treat infections did not occur until the 1940s, when Howard Walter Florey and Ernst Chain

developed biochemical methods to isolate and purify penicillin, making it possible to introduce this drug into mass distribution.

Due to the different chemical structure of β-lactam molecules and their respective antibacterial potency, semi-synthetic beta-lactam compounds were continuously and systematically developed.

Common features include the presence of the β-lactam ring linked to a thiazolidine ring (penicillins) or a dihydrothiazin ring (cephalosporins) and an identical mechanism of action as they are drugs with bactericidal action interfering with bacterial cell wall synthesis. This ring mimics the shape of the terminal D-Ala-D-Ala peptide sequence that serves as the substrate for cell wall transpeptidases that form covalent bonds between different peptidoglycan chains during periods of cell growth. (*Figure 2)*

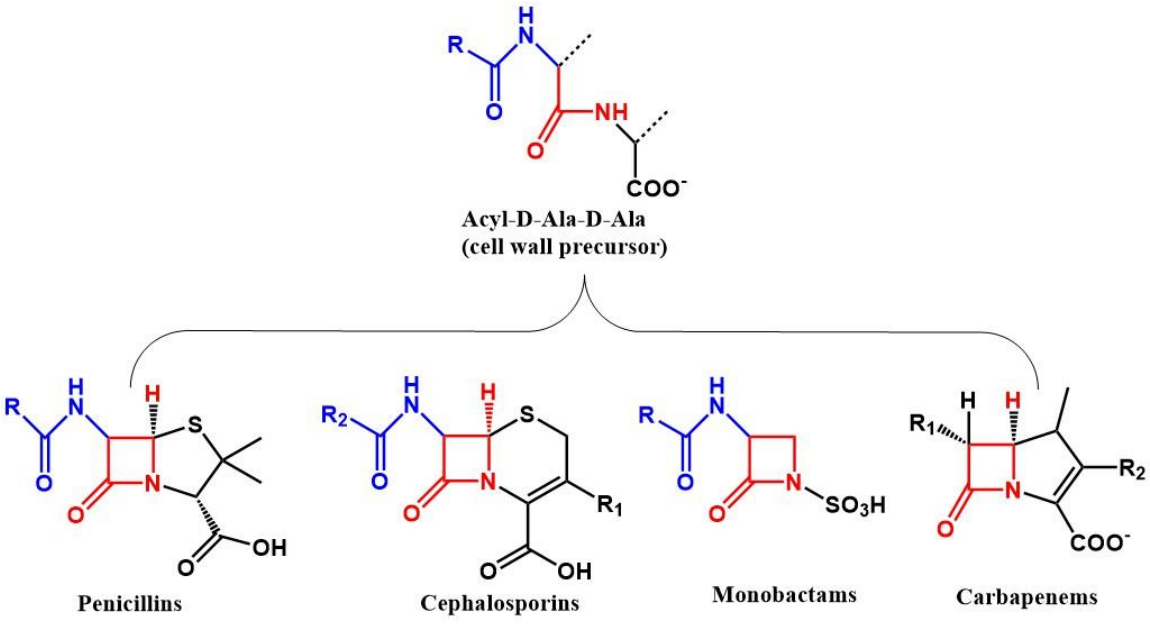

Figure 2. Structure and classification of β-lactam antibiotics (adapted from https://www.mdpi.com/1467-3045/45/1/7)

There are three enzymes' families located on the surface of the cytoplasmic membrane: trans glycosylase, transpeptidase and carboxypeptidase known under the acronym PBP (*Penicillin Binding Protein*), involved in the biosynthesis of peptidoglycan (PG), the main component of bacterial cell walls. They represent the elective target for β-lactams, which act through a competitive mechanism by selectively inhibiting each PBP.

Among β-lactams, carbapenems are those with the broadest activity spectrum of bacterial activity, being able to resist several β-lactam- inactivating enzymes (i.e. β-lactamases). They have a structure very similar to penicillins, with the modification of the typical thiazolin ring, in that the nitrogen atom in position 1 has been replaced with a carbon atom, hence the name carbapenems. Carbapenems are hydrolysed very slowly by most β-lactamases and for this reason, since their introduction in the 1980s, these antibiotics have been successfully employed and have been used against multidrug-resistant bacteria. Some examples are ertapenem, imipenem, meropenem.

1.2.2 Inhibition of replication, transcription and translation of nucleic acids

DNA topoisomerases are enzymes involved in the regulation of DNA metabolism, enabling the transition of DNA molecules from one topoisomer to another, by modifying the degree of supercoiling of the molecule itself, condensing or relaxing it, by acting on the number of times it is coiled upon itself. Two topoisomerases are important as molecular drug targets: DNA gyrase and DNA topoisomerase IV, both essential for bacterial chromosome replication.

DNA gyrase is a key enzyme in the DNA duplication process in bacteria. The rapid rotation of the double helix that occurs during duplication can cause supercoiling of

DNA strands, which prevents the continuation of the synthesis process; gyrase cuts and closes the supercoiled stretches, removing them.

DNA topoisomerase IV is crucial for the segregation of bacterial DNA during cell division and helps unwind interconnected DNA strands. *(Figure 3)*

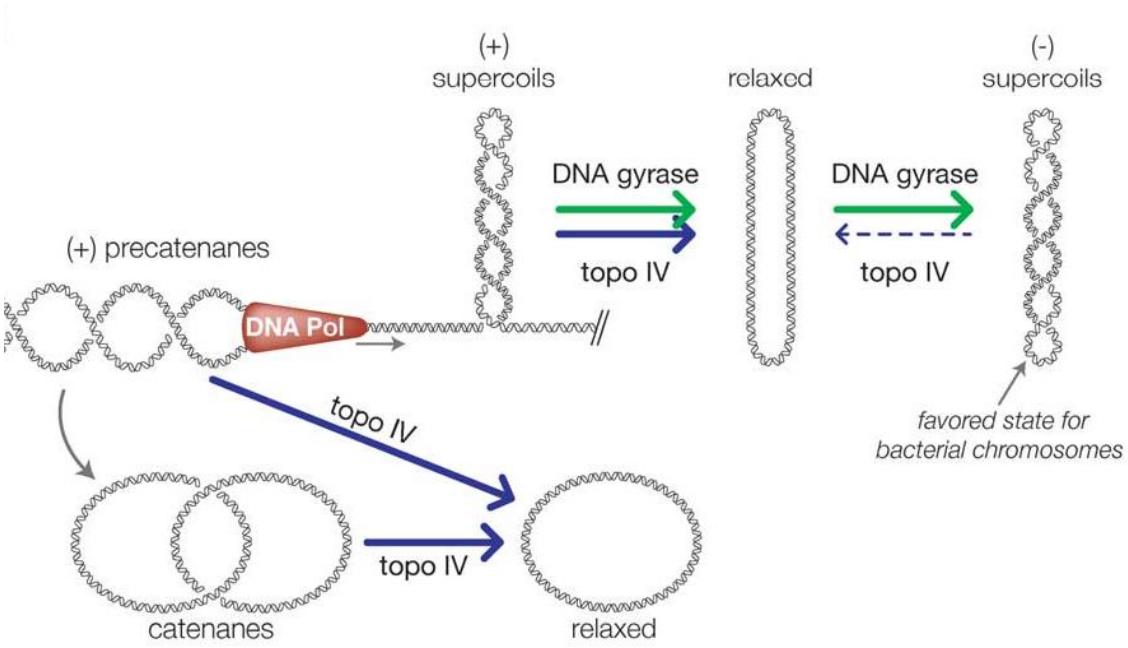

Figure 3. Reactions catalysed by DNA gyrase and topoisomerase IV (adapted from https://www.sciencedirect.com/science/article/abs/pii/S0022283605006790?via%3Dihub)

Transcription of the bacterial chromosome is carried out by RNA polymerase, which enables the synthesis of an RNA strand complementary to one of the two DNA strands that make up the double helix, and represents another therapeutic target.

Antibiotics can interfere with nucleic acid replication and transcription, thus damaging the genetic material essential for the bacteria growth, and finally blocking cell replication and survival. This class includes quinolones, fluoroquinolones, ansamycins.

Ribosomes play a key role in protein synthesis. In bacterial cells, they have a sedimentation coefficient of 70 Svedberg units (70S). They consist of two subunits, one small (30S) and one large (50S). There are antibiotics that hinder protein synthesis by acting on the 30S or 50S subunit of ribosomes. Proteins are essential products for the cell survival, so that blocking their macromolecular synthesis process directly results in bacterial killing. This class include aminoglycosides, tetracyclines, chloramphenicol, macrolides, lincosamides (clindamycin), streptogramins, oxazolidinones (linezolid).

1.2.3 Metabolism inhibition

Bacteria are not able to utilise pre-formed folic acid, unlike eukaryotic cells, which is essential for the synthesis of DNA, RNA and proteins, and therefore need to synthesise it from para-aminobenzoic acid (PABA) and pteridine which are bound by the enzyme dihydropteroate synthetase (DHPS). Sulphonamide blocks the enzyme and the reaction does not proceed. Bacteria that do not depend on the dihydrofolic acid biosynthetic pathway, such as *enterococci* and *lactobacilli*, are intrinsically resistant to sulphonamides.

Diaminopyrimidines have as their molecular target the enzyme dihydrofolate reductase, involved in the last step of tetrahydrofolic acid synthesis, catalysing the reduction of dihydrofolate to tetrahydrofolate.

These targets are not present, or are structurally different in humans, which means that antibiotics do not harm our cells. There are many different antibiotics that can cause many different side effects. Some classes of antibiotics possess their own 'typical' toxicity towards certain organs or tissues, as the ototoxicity induced by aminoglycoside antibiotics or the photosensitivity reactions typical of the tetracyclines.

A common contraindication to all antibiotics is hypersensitivity to the active ingredient of a specific antibiotic, or to the active ingredients of other antibiotics belonging to the same class. In this case, the intake of certain antibiotics may induce moderate allergic reactions, which can be extinguished with the use of antihistamines; in other cases, severe allergic reactions, i.e. anaphylactic reactions, occur.

1.3 Antibiotics resistance: general concepts

As antibiotics are primarily natural agents, antibiotic resistance exists for all known antibiotics included in the actual clinical pipeline. Over the past decades, extremely resistant pathogens have become more common in the clinical setting, representing a major human health threat. Bacteria can develop resistance to one or few antimicrobials using two main pathways:

- ✓ Emergence of random mutations affecting the bacterial DNA
- ✓ Acquisition of novel resistance genes through *horizontal gene transfer*

Very often, bacterial species are naturally sensitive to the activity of an antibiotic and, under certain conditions, lose sensitivity to concentrations of the drug that are therapeutically achievable *in vivo* in the human organism.

Emergence of DNA mutations affecting the coding sequence of the antibiotic target constitutes only 10-15% of all acquired resistance (low frequency of occurrence), and the mutated genetic character is transmitted vertically to daughter cells. It can also follow a step-wise development, when several mutations (acting in concert) are needed for the emergence of a resistance phenotype (e.g. fluoroquinolones), or one-step, when only one mutation is needed (e.g. rifamycins).

Of course, greater importance is embodied in the horizontally acquired resistance, which accounts for 90% of all resistance (high frequency of occurrence). It originates from the acquisition of new genetic information from other microorganisms via horizontal gene transfer events, mainly including conjugation (plasmids, transposons) and transformation (free DNA).

It is interesting to appreciate how, due to characteristics of the mobile genetic elements, such resistance often involves several classes of antibiotics (resistance is often associated with a phenomenon known as genetic linkage). This fact poses additional problems for the choice of the most suitable antibiotic therapy for the treatment of a given infectious disease. In particular, the transfer of resistance determinants is not species-specific (transfer between strains of the same species), but can also occur between microorganisms belonging to differently related species. Clearly, this behaviour contributes considerably to the spread of resistance and is a risk factor associated with the use of antibiotics, which should not be underestimated at all.

1.4 Genetic mechanisms underlying antimicrobial resistance

Antibiotic resistance is related to several molecular mechanisms that are used by bacteria to counteract the action of the antibiotic molecule; based on the specific mechanism involved, a classification in four main resistance groups has been defined.

1.4.1 Production of drug-inactivating /-modifying enzymes

The production of drug-inactivating/-modifying enzymes consists of the bacterium's ability to produce proteins with enzymatic activity capable of degrading or directly modifying the antibiotic, thus rendering it inactive. Antibiotics modified or inactivated by this mechanism are, for examples, aminoglycosides (by aminoglycoside-modifying enzymes) or β-lactams (by β-lactamases), respectively.

1.4.2 Overexpression of efflux pumps

In many bacteria, both gram-negative and gram-positive, there are active efflux systems, capable of expelling structurally different compounds from the bacterial cell, including multiple classes of antibacterial drugs (multidrug efflux systems MDR). As a consequence of mutational events leading to the increased expression of these active efflux systems, bacteria may develop a resistance phenotype agents. Another type of efflux systems is represented by systems that can only excrete specific classes of antibacterial drugs (drug-specific pumps). These systems, unlike multidrug systems, are generally plasmid-encoded and acquired by horizontal gene transfer. An antibiotic class affected by this mechanism is, for examples, that of fluoroquinolones.

1.4.3 Modification of drug permeability

Enterobacterales as Gram-negative bacilli are characterised by the presence of an outer lipopolysaccharide layer crossed by porins. The modification of membrane permeability is regulated by porins and, in particular, by changes in their expression; the production of specific porins able to prevent the penetration of the antibacterial agent can also be observed. As a result, for example, the β-lactams, no longer penetrate in sufficient quantities to interfere effectively with its molecular target, and to exert their inhibitory activity. Antibiotics affected by this mechanism are β-lactams, fluoroquinolones, tetracyclines and chloramphenicol.

1.4.4 Target alteration

The antibiotic targets can be altered, either by mutations in their coding sequences, or by enzymatic addition of chemical groups, both resulting in a reduced affinity of bond with the antibiotic. Also, the target can be produced in greater quantities, such that it is not sufficiently inactivated by the of the antibiotic. One example is represented by the DNA gyrases, targeted by fluoroquinolones; in this case, a missense mutation leading to the substitution of one or more amino acids is enough to make the enzyme very resistant to this class of drugs. Bacteria are able to resort to alternative metabolic pathways to those blocked by antibacterials. Another example is that of the dihydrofolate reductase enzyme, targeted by trimethoprim; in that case, the horizontal acquisition of an insensitive homologues of the (sensitive) dihydrofolate reductases can contribute to resistance.

1.5 β-lactamases

β-lactamases are a class of different enzymes that break the β-lactam ring, inactivating the β-lactam antibiotic. Some β-lactamases are encoded by genes located on mobile genetic elements (e.g. transposons, plasmids), while others are chromosome borne. The production of β-lactamases is among the most clinically relevant resistance mechanism to β-lactams in Gram-negative pathogens. Understanding the type of β-lactamases produced by different pathogens in some cases can help with susceptibility interpretation, therapeutic decision-making and infection control practices.

There are thousands of different types of β-lactamases, and multiple classification schemes are available. According to Ambler's classification, β-lactamases can be classified in four classes on the basis of their molecular homology: class A, C and D have a serine residue in the active site, whereas class B enzymes have zinc in the active site, i.e., metallo-β-lactamases. (*Figure. 4)*

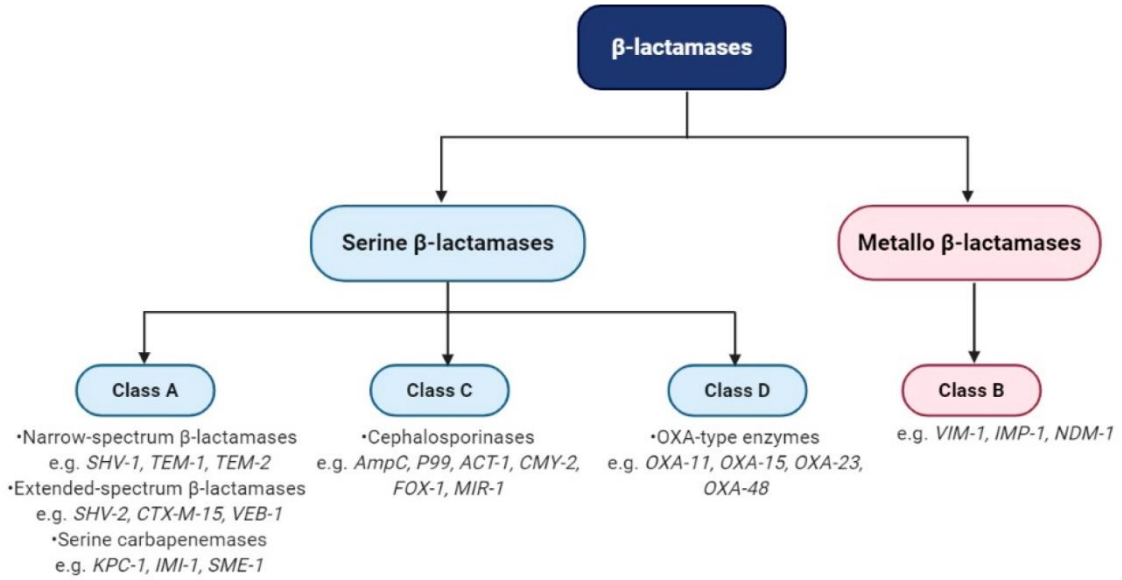

Figure 4. Ambler classification system of β-lactamases (adapted from https://www.mdpi.com/1422-0067/21/22/8527)

1.5.1 Class A β-lactamases

Class A comprises ESBLs (extended-spectrum β-lactamases), KPC (*Klebsiella pneumoniae* carbapenemases), IMI, and SME carbapenemases, penicillinases, IRT (inhibitor-resistance TEM), ESBL cephalosporinases (e.g. SHV, CTX-M, PER, VEB). From a clinical standpoint, ESBLs and KPC carbapenemases are the most relevant β-lactamases [2].

ESBLs are plasmid-encoded enzymes found mainly in *K. pneumoniae*, *Escherichia coli*, and other *Enterobacterales*. They can hydrolyse extended-spectrum penicillins (e.g. piperacillin), most cephalosporins (cephamycins are not hydrolysed by most extended-spectrum beta-lactamases) and monobactams. KPC are similar to ESBLs, except that they also hydrolyse carbapenemases. These enzymes first emerged in *K. pneumoniae* but have spread to other *Enterobacterales*. KPC enzymes are generally carried by transposons, so they can be transferred to different species, in fact genes encoding for KPC, initially identified in *Enterobacterales*, have also been found in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* group.

1.5.2 Class B β-lactamases (metallo-β-lactamases)

Metallo-β-lactamases, including VIM, IMP and NDM, use a zinc ion to hydrolyse all β-lactams, including carbapenems, except the aztreonam (monobactam). Metallo-β-lactamases (MBL) can be chromosomally encoded in some microorganisms, such as the L1 MBL of *Stenotrophomonas maltophilia*, or they can be horizontally acquired by a variety of Gram-negative microorganisms including *Klebsiella*, *Pseudomonas*, and *Acinetobacter*. These enzymes are not inhibited by currently available β-lactamase inhibitors.

1.5.3 Class C β-lactamases (AmpC)

Class C AmpC enzymes hydrolyse most cephalosporins (except cefepime), cephamycins (e.g. cefoxitin, cefotetan), monobactams (e.g. aztreonam) and penicillins. AmpC-type β-lactamases can be chromosome or plasmid-encoded. Notably, overexpression of chromosomal AmpC can be induced by certain antibacterials. Expression of plasmid-encoded AmpC is also constitutive and may be widespread among bacteria usually lacking this β-lactamase, such as *E. coli*, *K. pneumoniae*, and *Proteus mirabilis*. Isolates with inducible AmpC expression may initially be sensitive to 3rd generation cephalosporins, which may complicate treatment decisions, especially for *Enterobacterales.*

1.5.4 Class D β-lactamases (oxacillinases)

OXA β-lactamases (oxacillinases) hydrolyse mainly narrow-spectrum penicillins, but some variants such as plasmid-encoded OXA-48-like enzymes can hydrolyse carbapenems (responsible for carbapenemase resistance in *Acinetobacter spp*, for example) even leaving many cephalosporins active. Like the other carbapenemases, OXAs are not sensitive to the action of ß-lactamase inhibitors.

1.6 Carbapenem resistant *Enterobacterales* (CRE)

The order *Enterobacterales* includes several enteric Gram-negative bacilli that, occasionally, may act as human opportunistic pathogens, such as *K. pneumoniae* and *E. coli*, which are also prone to acquire AMR determinants [3]. Infections sustained by CRE are associated with significant high morbidity and mortality, and represent a

growing threat to public health worldwide [4]. As a result, CRE has been listed as one of the three most urgent antimicrobial-resistance threats by the Centers for Disease Control and Prevention (CDC) and as pathogens of critical priority by the World Health Organization (WHO) [5].

Bloodstream infections (BSIs) caused by CRE are associated with worse outcome compared to other sites of infections: the pooled mortality was much higher than urinary tract infection (UTI, 54.3% versus 13.52%) in patients with carbapenem-resistant *K. pneumoniae* [6].

Infections sustained by CRE can arise from many sites, including: the urinary tract, lungs, abdomen, surgical site, and bloodstream. Other risk factors include travel to an endemic area of CRE, immunocompromised state, mechanical ventilation, and advanced age.

Almost all of CRE bacteraemias diagnosed in 2021 were caused by *K. pneumoniae* (97.1%), and only a small part by *E. coli* (2.9%) [7]. In 2021, reports were sent in from 18 Regions/Autonomous Provinces; no cases were reported by Basilicata (which had not reported any cases in 2020) and Calabria (which had reported only 3 cases in 2020), while the data for Campania is not available as it did not send in reports. Overall, reports were received from 210 Hospitals/Health Agencies/Local Health Units, a decrease compared to 2020 when data were reported by 269 facilities [8].

Central Italy was the geographical area with the highest incidence of cases (IRst=4.3 per 100,000 residents) followed by the North (IRst=3.2 per 100,000 residents) and the South and Islands (IRst=2.5 per 100,000 residents).

The incidence of cases in Central Italy is stable compared to 2020 (IRst=4.3 per 100,000 residents). In contrast, the North confirms the increase in the incidence rate

already observed in 2019-2020 (IRst respectively 2.3 and 2.6 per 100,000 residents). The South and Islands show a decrease in the incidence rate compared to 2020 (IRst= 3.6 per 100,000 residents). (*Figure. 5)*

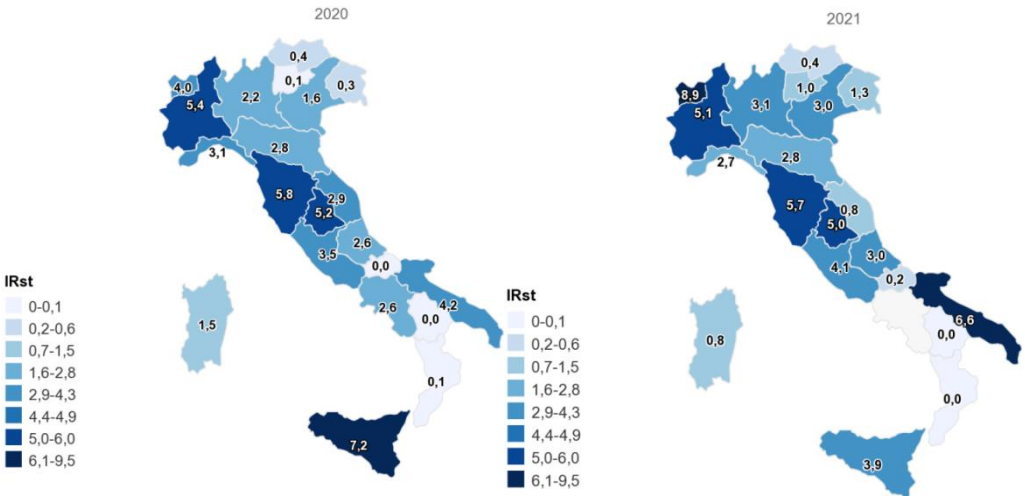

Figure 5. Regional age-standardised incidence rate per 100000 residents of diagnosed CRE bacteraemia cases in Italy 2020-2021 (adapted from https://www.epicentro.iss.it/antibiotico-resistenza/cre-dati)

1.7 Carbapenem-resistant *Klebsiella pneumoniae*

K. pneumoniae is an opportunistic pathogen that normally colonises the human gastrointestinal tract, skin and upper respiratory tract. It's Gram-negative bacterium, has a rod form and is the most important member of the genus *Klebsiella*. Over paste decades, *K. pneumoniae* showed a remarkable ability to developed resistance to several classes of antibiotics following horizontal acquisition of multiple resistance determinants, thus limiting the available treatment options. The production of class A β-lactamases, like KPC or ESBLs, makes the bacterium resistant to penicillins and narrow-spectrum cephalosporins. In recent years, the production of ESBLs capable of rendering broad-spectrum β-lactams ineffective, including third-generation

cephalosporins, has increased the use of carbapenems and thus favoured the rapid spread of resistance to the latter class of antibiotics [9].

Carbapenemes resistance is now a major public health problem and often arises in strains already resistant to other classes of antibiotics *(Figure 6)*. Several types of carbapenemases have been identified in *K. pneumoniae*, including KPC- and OXA-48-like and NDM-type enzymes [10].

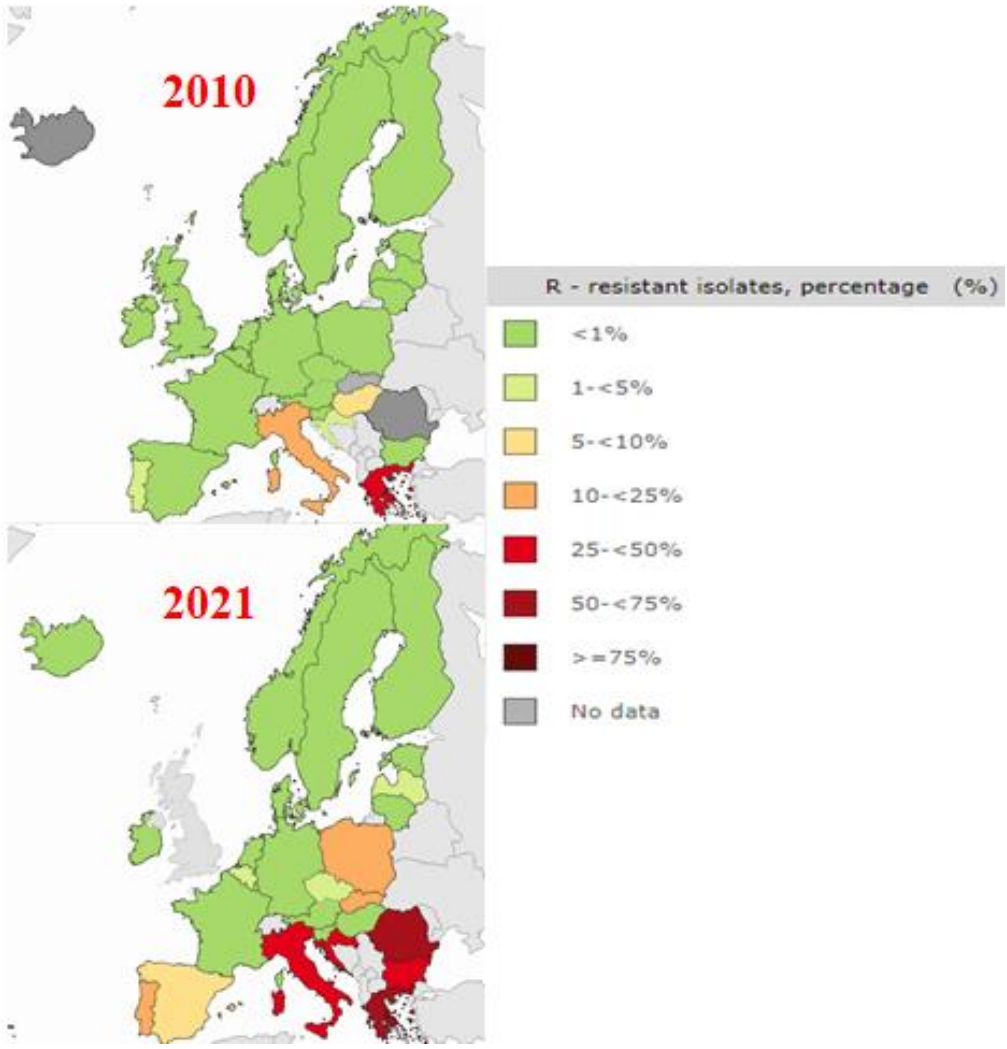

Figure 6. Carbapenemase resistance rates in Klebsiella pneumoniae isolates in Europe 2010-2021 (adapted from https://atlas.ecdc.europa.eu/public/index.aspx)

They efficiently hydrolyse penicillins, all cephalosporins, monobactams, carbapenems, and even β-lactamase inhibitors, like clavulanic acid or tazobactam. . Since their first

description, these enzymes have spread across countries and continents, although the exact epidemiology of their expansion varies by geographical location.

KPC, first isolated in 1996 from *K. pneumoniae*, in the USA is mainly detected in *K. pneumoniae* [11]. It is widespread in the USA, South America, China, Israel, Greece. In Italy it has a high endemicity, generally from previously hospitalised patients [12].

In Europe, KPC-producing *K. pneumoniae* prevalence is around 45%, with higher percentages in Italy and lower percentages in Belgium. In Italy, after an increase observed in 2019-2020, a decrease in the proportion of KPC isolates was observed in 2021 (26.7% vs. 29.5% in 2020). According to national surveillance data on bacteraemia caused by CRE, the most widespread pathogen is *K. pneumoniae* producing KPC enzyme. In 2021 the incidence is 80.6% [13].

OXA-48 (oxacillinase 48), first isolated in 2001 from *K. pneumoniae* in Turkey [14]. Prevalent in Turkey, Middle East and North Africa. Less frequent in Italy. OXA-48-like enzymes are the most prevalent carbapenemases among *Enterobacterales* in much of Western Europe. They are most frequently detected in *K. pneumoniae* and *E. coli* but may be associated with other *Enterobacterales.*

In Europe, OXA-48 prevalence is around 37%, with higher percentages in Turkey and lower percentages in UK. In Italy in 2021 there is a low incidence rate of 1.9%.

VIM (Verona imipenemasis), first isolated in 1997 from *P. aeruginosa* in Italy [15]. Widespread throughout the world, in Europe we find it mainly in Greece and Cyprus.

In Europe, VIM prevalence is around 8%, with higher percentages in Hungary and lower percentages in France. In Italy in 2021 there is a low incidence rate of 1.8%.

NDM (New Delhi metallo-ß-lactamase), first isolated in 2009 from *K. pneumoniae* in Swedish patient previously admitted in New Delhi [16]. As for epidemiology, great diffusion in India, Pakistan, Bangladesh, Balkans, UK. In Italy only single local epidemics, sporadic isolates [17].

In Europe, NDM prevalence is around 11%, with higher percentages in Serbia and lower percentages in Portugal, for example. In Italy in 2021 incidence rate is 10.6%.

1.8 The novel anti-CRE agents

One of the main strategies used to restore the effectiveness of β-lactam antibiotics is to use β-lactamase inhibitory molecules to prevent the antibiotic from being hydrolysed by the enzyme.

In recent years, indeed, novel β-lactam antibiotic/β-lactamase inhibitor combinations (BLICs), including ceftazidime/avibactam (CAZ/AVI), imipenem/relebactam and meropenem/vaborbactam have been introduced into clinical practice for the treatment of infections caused by carbapenemase-resistant *K. pneumoniae*, although resistant strains have already been isolated for these new molecules and different types of resistance mechanisms described.

Ceftazidime/avibactam (CAZ-AVI), a cephalosporin paired with a novel diazabicyclooctane inhibitor, is the combination of a third-generation cephalosporin with a non-β-lactam β-lactamase inhibitor, recommended in the treatment of complicated intra-abdominal infections, acute pyelonephritis and complicated urinary tract infections as well as hospital-acquired pneumonia (HAP), including ventilator-associated pneumonia (VAP) [18].

Avibactam is able to inhibit class A β-lactamases (TEM, SHV, CTX-M), KPCs, chromosomal AmpCs and CMY plasmid variants and some class D β-lactamases, such as OXA-48. Unfortunately, Avibactam is not active towards metallo-β-lactamases. In *Acinetobacter* spp. resistance to CAZ/AVI is mainly due to the failure of Avibactam to penetrate the intracellular compartment. Several mechanisms of CAZ/AVI resistance have been described (increased beta-lactamase and/or of efflux pumps activity, functional alterations of porins, point mutations in penicillin-binding proteins). With regard to KPC-producing *K. pneumoniae*, resistance to CAZ/AVI may be due to increased expression of KPC or by emergence of novel KPC variants that can lead to loss of Avibactam inhibition, resulting in the acquisition of resistance to CAZ/AVI. In these cases, the mutated KPC enzyme often results in a reduced ability to hydrolyse carbapenems, resulting in a peculiar antibiotic resistance phenotype that mimics the phenotype of ESBL-producing strains.

Meropenem/vaborbactam (MER/VAB) (a carbapenem paired with non-β-lactam β-lactamase inhibitor derived from boronic acid) was developed to restore meropenem's activity against carbapenemase-producing bacteria, and it is indicated for the treatment of complicated urinary tract infections, including pyelonephritis, complicated intra-abdominal infections, nosocomial pneumonias (HAP), and ventilator-associated pneumonias (VAP).

Vaborbactam inhibits both class A (KPC, IMI, SME, NMC-A, and FRI-1) and class C (AmpC, CMY) serine β-lactamases. With *P. aeruginosa* and *Acinetobacter* spp. the MER/VAB activity was overall similar to that of meropenem alone. This is apparently due to the fact that, in *P. aeruginosa* and *Acinetobacter* spp., resistance to meropenem is largely mediated by mechanisms that are not antagonised by Vaborbactam (e.g. membrane impermeability, efflux pumps and production of class B or D β-lactamases).

The combination of imipenem-cilastatin with relebactam (IMI/REL), a carbapenem paired with a novel diazabicyclooctane inhibitor, was mainly developed to restore activity against class A β-lactamases (KPC) and class C β-lactamases, with no activity against metallo-β-lactamases (e.g IMP, VIM and NDM) and OXA-48-like enzymes, and it is indicated for the treatment of hospital-acquired pneumonia and pneumonia associated with mechanical ventilation (VAP), the treatment of bacteremia occurring in association with HAP or VAP. In KPC-producing *Enterobacterales*, resistance to IMI/REL is mainly caused by mutations or overexpression of the KPC enzyme and/or functional alterations of the OmpK36 and OmpK35 porins. With regard to *P. aeruginosa*, the major determinants of resistance to IMI/REL are the presence of GES enzymes with carbapenemase activity (especially GES-5) and the loss of the OprD porin.

1.9 Aim of the thesis project

The aim of this study is to investigate the molecular bases of CAZ/AVI resistance in a collection of *K. pneumoniae* isolates of clinical origin collected at the San Martino Hospital (Genoa)

2 MATERIALS AND METHODS

2.1 Bacterial isolates

In this study, clinical isolates of *K. pneumoniae* resistant to CAZ/AVI, obtained from patients hospitalized at the Policlinic San Martino Hospital in Genoa admitted from 2019 to 2023 were analyzed (n= 12).

Twelve strains of *K. pneumoniae* from different sampling sites were analyzed, who showed a resistance phenotype to CAZ/AVI (MIC>8 μg/mL) in the diagnostic routine by the Clinical Microbiology laboratory of Policlinico San Martino Hospital Genoa.

For precise and unambiguous identification each strain has been classified by a collection code, year of collections, sampling source, resistance mechanism to carbapenems. *(Table. 1)*

Collection	**Year**	**Microorganism**	**Code**	**Source**	**Carbapenem Resistance mechanism**
E140	2023	*K. pneumoniae*	EMO0260589	blood culture	KPC
E187	2019	*K. pneumoniae*	EMO0247105	blood culture	KPC
E260	2021	*K. pneumoniae*	EMO0380985	blood culture	KPC
E469	2019	*K. pneumoniae*	EMO0258106	blood culture	KPC
E481	2020	*K. pneumoniae*	EMO0286795	blood culture	KPC
E1140	2021	*K. pneumoniae*	EMO0416083	blood culture	KPC
NE36	2020	*K. pneumoniae*	URO0151002	Urinoculture	KPC
NE87	2023	*K. pneumoniae*	LBI0658429	Sputum	KPC
NE129	2020	*K. pneumoniae*	URO0158109	Urinoculture	KPC
NE165	2019	*K. pneumoniae*	TAM0029593	Rectal Swab	KPC
NE497	2022	*K. pneumoniae*	TAM0211636	Rectal Swab	KPC
NE704	2021	*K. pneumoniae*	TAM0256985	Rectal Swab	KPC

Table 1. Characteristics of K. pneumoniae isolates investigated in this study.

2.2 Storage of bacteria isolates and culturing conditions

In the microbiology research laboratory, there are collections of pure cultures of microorganisms, which can be stored for long periods mainly through the technique of cryopreservation.

The main objective of this technique is to ensure that the culture remains viable and genetically stable over time. Cryopreservation can be accomplished by basic microbiology equipment, such as a laminar flow hood to cultivate bacteria in sterility, an autoclave to provide sterilization of growth media, and a refrigerator that reaches a temperature of at least -20°C.

All bacterial isolates were stored at -80°C in Brain Heart Infusion broth (BHI) (Oxoid, Hampshire, UK) containing 20% v/v glycerol (Sigma Aldrich, Saint-Louis, Missouri, USA): BHI broth is a general purpose medium used for the isolation, cultivation, and maintenance of a variety of fastidious and nonfastidious microorganisms, is a highly nutritious base that meets the growth requirements of many types of microorganisms including bacteria, yeasts, and mold. The glycerol stocks were used as sources of the isolates for all the experiments performed in this study. From frozen stocks, bacteria were plated on Mueller-Hinton Agar (MHA) plates (Oxoid, Hampshire, UK) and broth (200-220 rpm shaking), and incubated overnight at 37 °C prior to downstream analyses (i.e., genomic DNA extraction, antimicrobial susceptibility testing) [19].

Mueller Hinton broth is a liquid medium recommended for quantitative antimicrobial susceptibility testing of common, rapidly growing aerobic and facultatively anaerobic bacteria by broth dilution procedures, as standardized by the Clinical and Laboratory Standard Institute (CLSI). This medium is formulated to have low levels of thymine and thymidine and is adjusted to the calcium and magnesium ions concentrations recommended in CLSI document M7.

Once isolated colonies have been obtained on the culture plates, it is possible to have, already from their direct observation, an idea of the type of bacterium based on their

shape, colour and smell of them, as well as any changes in the medium around the growth (haemolysis, opacification, change in original colour, etc.).

2.3 Species identification

Species identification was performed using 'Matrix-Assisted Laser Desorption/Ionisation - Time of Flight' (MALDI-ToF). MALDI-ToF actually is the reference method used in clinical microbiology laboratories for species identification. Like all mass spectrometers, the MALDI-ToF consists of a component called the 'source' (MALDI), which generates the analytes (analysed molecules), and a component called the 'analyser' (ToF), which processes and compares the data produced by the analytes. In order to obtain the mass spectrometry output, it will be necessary to compare the data obtained experimentally with the data stored in specific databases, so as to be able to return analysis data with a variable degree of reliability. In the microbiological application, the output of this spectrometry is the identification of bacterial and fungal species.

2.4 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) is a phenotypic laboratory test used to determine the susceptibility of microorganisms to different antibiotics; it is an essential guide to therapy because it makes possible to choose the most appropriate antibiotic agents for the individual case.

In this project, AST was initially determined through the Vitek 2 (bioMerieux,

France) automated system, to select strains with resistance to CAZ/AVI.

Then, the results were confirmed using reference broth micro-dilution (BMD), considered the gold standard method.

Tested antibiotics included: meropenem, ceftazidime, ceftazidime/avibactam (avibactam at a fixed concentration of 4 µg/mL). This test employs disposable 96-well plates containing scalar dilutions of the different active ingredients assayed. It is performed with Mueller-Hinton broth containing serial dilutions of meropenem, ceftazidime and CAZ/AVI, using concentrations ranging from 0.25 to 256 µg/mL. *E. coli* ATCC 25922 was used as quality control strain. *(Figure. 1)*

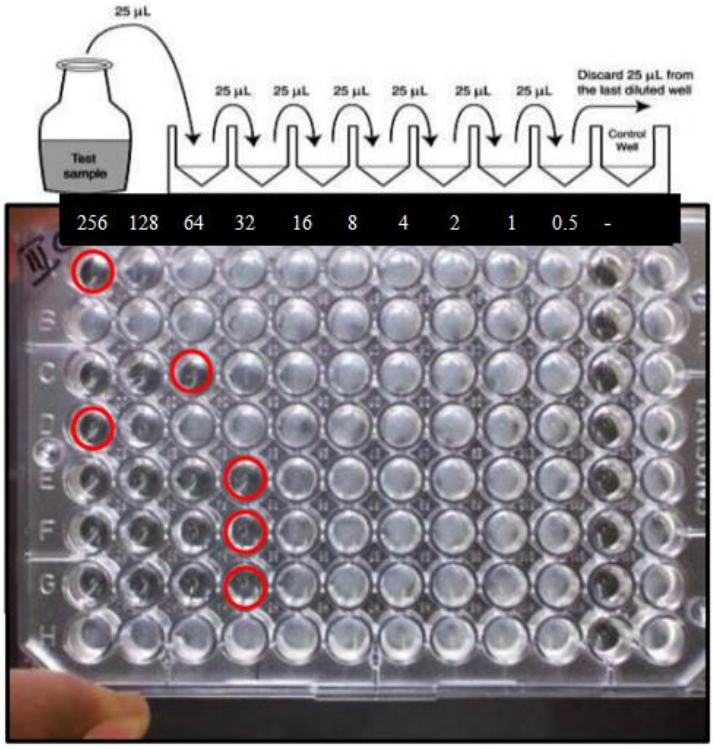

Figure 1. AST by reference broth microdilution (adapted from https://www.seremi.it/sites/default/files/2018-2019%20Corso%20FAD%20AMR_3_%20Antibiogramma.pdf)

In the AST, the susceptible (S), susceptible by increased exposure (I) and

resistant (R) categories are given for each microorganism/antibiotic combination according to interpretation criteria provided by EUCAST for the Minimum Inhibitory Concentration (MIC), which represents the lowest concentration expressed in μg/mL of antibiotic capable of inhibiting the growth of a given bacterial strain.

- ✓ S, Susceptible, standard dosage regimen: a microorganism is classified as 'S' when there is a high probability of therapeutic success using a standard dosage regimen of the antibiotic;
- ✓ I, Susceptible, increasing exposure: a microorganism is categorized as I' when there is a high probability of therapeutic success because the exposure to the antibiotic is increased by adjustment of the dose or concentration at the site of infection;
- ✓ R, Resistant: a micro-organism is classified as 'R' when there is a high probability of therapeutic failure, even in the presence of increased exposure.

EUCAST is the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org/). It was established in 1997 by members of ESCMID (European Society of Clinical Microbiology and Infectious Disease - www.escmid.org/) and has the precise aim of providing up-to-date breakpoints for the reading of antimicrobial susceptibility tests, whether automated or manual, by microbiology laboratories across Europe [20].

The crucial function of this organization is to provide standardization in the reading of these tests, by providing a network of knowledge compiled by

experts, reflecting the European situation at the microbiological level. The commission was established in the field of human clinical microbiology.

For each type of microorganism, EUCAST identifies a number of active antimicrobial agents that must be tested, because they are considered to be the most appropriate for that bacterial species and because they are less likely to induce dangerous resistance phenotypes.

2.5 Genomic DNA extraction

Total genomic DNA was extracted from bacterial cultures grown on MHA plates, following this protocol:

- ✓ 1 mL of ultrapure water was placed in a 2 mL eppendorf tube;
- ✓ 2-3 micro-organism colonies were taken with a 10 µL disposable loop and added to the eppendorf tube;
- ✓ vortexing;
- ✓ spinning at 14000 rpm for 2 min;
- ✓ the supernatant was eliminated;
- ✓ pellets were suspended in 200 µL of ultrapure water;
- ✓ placed the eppendorf tube in the termomixer at 95°C for 15 min;
- ✓ spinning at 14000 rpm for 2 min;
- ✓ supernatant was collected.

2.6 Real-Time PCR

In this study, the presence of genes coding for major carbapenemases, (including bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{OXA-48}) was performed by Real-Time PCR. DNA is amplified by DNA-polymerase chain reactions. After each round of amplification, the DNA is quantified. In a Real-Time PCR reaction, the amount of product formed is monitored during the entire reaction by measuring the fluorescence intensity emitted by particular fluorophores (acting as reporters) at each cycle. These fluorescent reporters will only emit visible light when they associate with the amplicon of the target sequence, so changes in the fluorescence emitted are a direct expression of the amount of product formed, allowing the number of target DNA molecules present at the start of the reaction to be quantified.

Furthermore, the bla_{KPC} gene copy-number was determined by relative RT-PCR.
The bla_{KPC} gene dosage was assessed by a relative qPCR quantification: estimates of the gene dosage were evaluated using the $\Delta\Delta_{CT}$ method, using as reference a CAZ/AVI susceptible KPC-producing *K. pneumoniae* strain, which carries a single copy of the bla_{KPC} gene.

2.7 Assessment of carbapenemase activity

The carbapenemase activity was determined as meropenem-hydrolyzing specific activity using a spectrophotometric assay on crude cell extracts prepared from culture grown (i.e., at 37°C, 200-220 rpm shaking) to mid-exponential-phase (i.e., OD_{600nm}:~0.5) in LB broth.

The carbapenemase activity was monitored by following the hydrolysis (λ=300) of 150 µM meropenem (substrate) in 50 mM HEPES buffer (Sigma Aldrich, Saint-Louis, US) for 2 minutes. The total protein content in crude extracts was determined by the Invitrogen Qubit 4 Fluorometer in order to calculate the carbapenemase specific activity (nmol of meropenem/min/mg of protein).

RESULTS

3.1 Outline of the CAZ/AVI resistant *K. pneumoniae*

Twelve (12) CAZ/AVI resistant (CAZ/AVI^{R}) *K. pneumoniae* were isolated from in-patients admitted at Policlinico San Martino Hospital, Genoa, from January 2019 to January 2023. The patients had a heterogeneous length of stay, with admissions often overlapping in different wards.

All twelve CAZ/AVI^{R} *K. pneumoniae* were positive for the *bla*$_{KPC}$ carbapenemase gene, and negative for genes encoding metallo-β-lactamases, accoding to Real-Time PCR results.

3.2 Antimicrobial susceptibility testing (AST)

The initial AST of the 12 *K. pneumoniae* isolates using the Vitek automated system revealed that all exhibited a difficult-to-treat resistance phenotype, being resistant to amoxicillin/clavulanic acid (>16 mg/L), amikacin (>32 mg/L), ciprofloxacina (>2 mg/L), piperacillin/tazobactam (>64 mg/L).

AST by reference broth microdilution confirmed the CAZ/AVI resistance (MIC>8 mg/L) phenotypes for 9/12 isolates, which showed a variable resistance to meropenem. (*Table 1*)

K. *pneumoniae* strain	MIC (mg/L)	
	meropenem	CAZ/AVI
E1140	128	64
E140	256	16
E187	2	32
E260	512	64
E469	256	32
E481	128	8
NE87	256	32
NE129	128	16
NE165	256	8
NE36	256	8
NE497	256	16
NE704	512	16

Table 1. Results from AST (meropenem and CAZ/AVI) performed on the K. pneumoniae isolates characterized in this study: in green the values associated with susceptible, in red those associated with resistant phenotype

In E481, NE165 and NE36 strains, resistance to CAZ/AVI was not confirmed by the AST performed by reference broth microdilution, so that they were not subjected to further analysis concerning the characterization of the resistance mechanism to CAZ/AVI.

As for meropenem, the highest MIC was 512 mg/L from sample E260, while the lowest MIC was 2 mg/L from sample E187; the modal MIC value was 256 mg/L. *(Fig 1)*

As for CAZ/AVI, the highest MIC was 64 mg/L from sample E1140 and E260, while the lowest MIC was 8 mg/L from sample E481, NE165, NE36. *(Figure. 1)*

The sample E260 had the highest MIC values for both meropenem and CAZ/AVI.

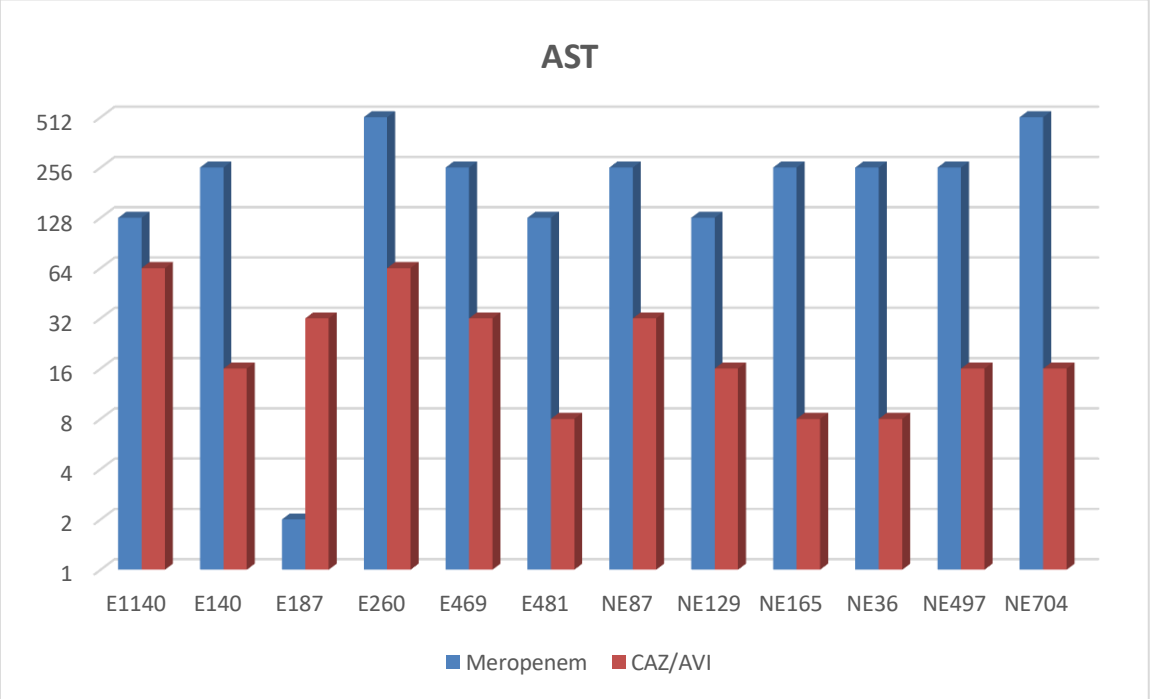

Figure 1. Antimicrobial susceptibility testing for meropenem and CAZ/AVI

3.3 Characterization of the CAZ/AVI resistance mechanisms

To characterize the mechanism of resistance to CAZ/AVI, we assessed the bla_{KPC} gene copy number by Real-Time PCR, and evaluated the enzymatic activity on meropemem.

According to the Real-Time PCR experiments, a significantly high bla_{KPC} gene dosage was detected in most isolates, compare to a reference KPC-encoding *K. pneumoniae* testing susceptible to CAZ/AVI. *(Figure. 2)*

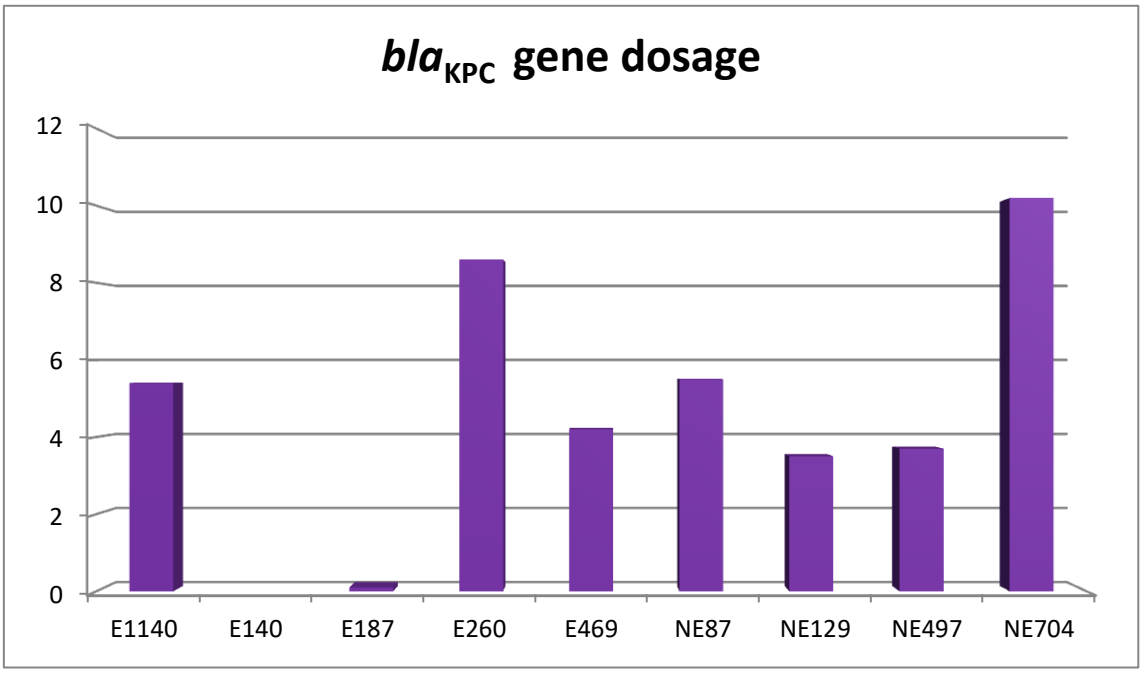

Figure 2. bla_{KPC} gene dosage assessed through Real-Time PCR experiments.

3.4 Carbapenemase activity

Quantification of the carbapenem-hydrolysing specific activity (nMol/mg^{-1}min^{-1}) using meropenem as substrate appears to be high in most samples

compare to a reference KPC-encoding *K. pneumoniae* testing susceptible to CAZ/AVI. *(Figure.3)*

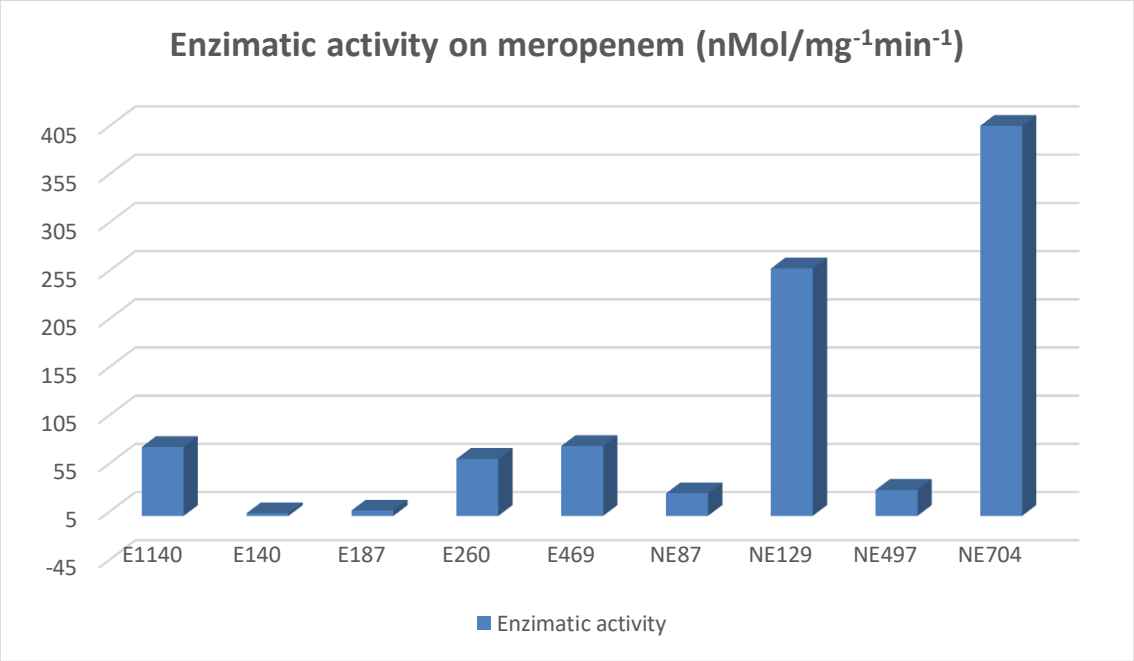

Figure 3. Evaluation of the meropenem-hydrolysing activity (nMol/mg^{-1}min^{-1})

One strain (E187) showed no activity toward meropenem, suggesting the presence of an enzymatic variant of KPC specialized in degrading CAZ/AVI but not meropenem.

One strain (E140) with *bla*$_{KPC}$ copy values and enzyme activity inconsistent with MIC values probably has a mechanism not yet characterized.

4 DISCUSSION

Novel BLICs have represented a major break-through in the treatment of difficult-to-treat infections caused by CPE-producing, KPC-type carbapenemases. However, resistance to novel BLICs, mostly CAZ/AVI, has been reported and represents a matter of notable concern.

The present study reported the characterization of nine CAZ/AVI resistant KPC *K. pneumoniae* from Policlinico San Martino Hospital, Genoa.

In most cases, CAZ/AVI resistance was associated with an increased KPC production owing to an increased copy number of bla_{KPC}, likely reflecting the multimerization of the bla_{KPC}-harbouring transposon, which was actually the most prevalent mechanism. However, it should be noted that a single case of KPC-*K. pneumoniae* (KPC-Kp) resistant to CAZ/AVI showed no activity toward meropenem, likely suggesting the presence of an enzymatic variant of KPC able to resist CAZ/AVI but not meropenem. Sanger sequencing could be considered to determine which KPC enzyme variant is present in this strain.

Another strain (E140), showing bla_{KPC} copy values and enzyme activity inconsistent with MIC values, has a mechanism of resistance to CAZ/AVI not yet characterized. In this case, it would be interesting to use whole genome sequencing to decipher the putative CAZ/AVI resistance mechanism.

The emergence of KPC overproduction as a resistance mechanism to new BLICs also has remarkable implications on the diagnostic side. In fact, following the introduction of novel BLICs, the rapid identification of carbapenemase genes in clinical specimens using molecular tests with syndromic panels has become crucial to tailor antimicrobial therapy based on

resistance determinants. However, current diagnostic platforms cannot identify enzyme overproduction; therefore, inferring susceptibility to new BLICs based on bla_{KPC} gene detection could be misleading in the presence of strains with this resistance mechanism. Therefore, this highlights the role that phenotypic antibiogram plays in the identification of resistance to novel BLICs, while waiting for next-generation molecular tests suitable for more accurate prediction of resistance profiles.

5 CONCLUSIONS

In conclusion, KPC overproduction can be an emerging mechanism of cross-resistance to CAZ/AVI in major clonal lineages of *K. pneumoniae*. The emergence of KPC *K. pneumoniae* strains with this resistance mechanism deserves major attention because it can potentially mediate cross-resistance to different BLICs, posing further limitations to the use of these novel compounds and leaving very few options for antimicrobial therapy. Global surveillance studies are warranted to monitor the evolution of the resistance traits of CRE, possibly coupled with a genomics-informed understanding of the mechanisms of resistance to novel BLICs.

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