Universidade de Lisboa
Faculdade de Farmácia

Exploring resistance levels to third-generation cephalosporins and carbapenems and its distribution: a correlation with the genetic background and molecular determinants

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Monografia orientada pelo Professor Doutor João Ruben Lucas Mota Perdigão, Professor Investigador Júnior

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Abstract

The emergence of strains resistant to different antibiotics has been increasing in the last few years. The increasing therapeutic failure due to the rise in bacterial resistance to a large number of different classes of antibiotics demonstrates the importance of studying and characterizing the evolution of drug resistance and its correlation with its molecular determinants.

This work aims to study the resistance levels towards different third-generation cephalosporins and carbapenems, by *Klebsiella pneumoniae*, compare different methodologies, their congruence and correlate these with molecular determinants of resistance.

A total of 106 isolates were included in the study, for which, the minimum inhibitory concentration (MIC) was determined for meropenem (MER), ceftazidime (CTZ), and cefotaxime (CTA) by broth microdilution along with the distribution of the diameter of inhibition zones (IZ) obtained by Kirby-Bauer disk diffusion. Also, the IZs for imipenem (IMI) and ertapenem (ERT) were determined by Kirby-Bauer disk diffusion with the aim of comparing the sensitivity of disk diffusion using drugs within the same drug class (carbapenems). The results showed that for CTZ and CTA, the majority of the clinical isolates, presented higher levels of resistance, meanwhile, MER demonstrated that most strains were susceptible to this antibiotic. Overall, both methods demonstrated similar results.

Regarding the molecular determinants of resistance, it is known that extended spectrum beta-lactamases (ESBL) and carbapenemases (CARB) are among the main enzymes that mediate bacterial resistance to cephalosporins and carbapenems and those are encoded in the genomic sequence of the bacterial strain. Using an AMRFinder tool and the NCBI Bacterial Antimicrobial Resistance Reference Gene Database, the identification of these resistance genes was possible, in which, among the 106 clinical isolates, 53 and 20 strains had ESBL, and CARB genes encoded in their genome, respectively. The most frequent genes observed were the *bla*$_{CTX\text{-}M\text{-}15}$, *bla*$_{KPC\text{-}3}$, and *bla*$_{TEM\text{-}10}$.

Key Words: *Klebsiella pneumoniae*; Susceptibility; Meropenem; Ceftazidime; Cefotaxime.

Resumo

O aparecimento de novas estirpes resistentes aos antibióticos tem vindo a aumentar nos últimos anos. Cada vez mais há falência terapêutica devido ao aumento da resistência das bactérias a um grande número de antibióticos pertencentes a diferentes classes e, por isso é importante estudar e caracterizar a evolução dessa resistência e a sua corelação com determinantes moleculares.

O objetivo deste estudo é tentar perceber os níveis de resistência a diferentes cefalosporinas de terceira geração e aos carbapenemos, da *Klebsiella pneumoniae*, comparar diferentes metodologias, a sua congruência e correlacioná-las com os determinantes moleculares de resistência.

Um total de 106 isolados foram incluídos no estudo, para os quais, foi determinada a concentração mínima inibitória (MIC) para o meropenem (MER), a ceftazidima (CTZ) e a cefotaxima (CTA) através do ensaio de microdiluição (BMD) e as suas zonas de inibição (IZ), pelo método de difusão em disco (DD). Além disso, as zonas de inibição para o imipenem (IMI) e o ertapenem (ERT) foram determinadas com o objetivo de comparar a sensibilidade do método de difusão em disco usando fármacos pertencentes à mesma classe (carbapenemos). Os resultados demonstraram que a maioria das estirpes apresentavam resistência à CTZ e à CTA, enquanto, por outro lado, a maioria dos isolados apresentavam-se suscetíveis ao MER. No geral, os resultados obtidos nos dois métodos eram maioritariamente concordantes.

Em relação aos determinantes moleculares de resistência, sabe-se que as beta-lactamases de largo espectro (ESBL) e as carbapenemases (CARB) estão entre as principais enzimas que conferem resistência bacteriana às cefalosporinas e carbapenemos e que são codificadas na sequência genômica de cada estirpe bacteriana. Utilizando a ferramenta AMRFinder e o NCBI *Bacterial Antimicrobial Resistance Reference Gene Database*, foi possível a identificação desses genes de resistência, em que, de entre os 106 isolados, 53 e 20 estirpes tinham genes que codificavam para ESBL e CARB no seu genoma, respetivamente. Os genes mais frequentes observados foram o *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{KPC-3}}$ e *bla*$_{\text{TEM-10}}$.

Palavras-Chave: *Klebsiella pneumoniae*; Suscetibilidade; Meropenem; Ceftazidima; Cefotaxima.

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Abbreviations List

AST – Antimicrobial Susceptibility Testing

BMD - Broth Microdilution

CA - Categorical Agreement

CARB - Carbenicillin Hydrolyzing Beta Lactamases

CRE - Carbapenem-resistant Enterobacteriaceae

CTA - Cefotaxime

CTZ - Ceftazidime

DD - Disk Diffusion

ERT - Ertapenem

ESLBs - Extended Spectrum Beta-Lactamases

EUCAST - European Committee for Antimicrobial Susceptibility Testing

IMI - Imipenem

IZ - Inhibition Zone

MDR - Multidrug Resistance

ME - Major Error

MER – Meropenem

MHA – Muller Hinton Agar

MIC - Minimal Inhibitory Concentration

MRSA - Methicillin-resistant Staphylococcus aureus

KP - *Klebsiella pneumoniae*

VME - Very Major Error

XDR - Extensively Drug-Resistant

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1. Introduction

1.1. Brief Historical Perspective of Antimicrobial Drugs and Drug Resistance

Antibiotics are among the most successful drugs ever developed, however, soon after their implementation, to therapeutic use, evidence that bacteria could develop mechanisms to overcome their effect was evident (1). These mechanisms allowed bacteria to survive when in presence of an antibiotic making the bacteria resistant to it. Such bacterial resistance phenomena was evident since the first antibiotic ever discovered, salvarsan, that was widely used until the implementation of penicillin. Salvarsan was discovered recurring to a library of synthetic compounds and was inspired by Paul Ehrlich's work on dyes a specifically stained bacterial cells. Despite not being used in clinical practices nowadays, salvarsan inspired the discovery of a different class of antibiotics, the sulfonamides (2). Around the same time, penicillin was discovered, laying the foundations for the development of newer antibiotics, not only, because of its beta-lactam structure that enabled the development of semi-synthetic derivatives to bypass penicillin resistance, but also, because it allowed scientists to realize that some microorganisms produced antimicrobial compounds that could be used in clinical settings as antibiotics. Therefore, after the discovery of penicillin the golden age of antibiotics discovery took place with the rapid discovery of multiple classes of naturally produced antibiotics during a relatively short period (3). In parallel with such rapid antibiotic discovery, excessive use of antibiotics started to occur over 1940s to 1960s and continued up to the present day. This, coupled with the lack of antibiotic discovery after, around the 1970s, led to the current situation with few new antibiotics in clinical trials (4) (5). Despite the antibiotic era, where infectious diseases were believed to be eradicated, scientists realized that this was not true because resistance strains were increasing rapidly (6).

Soon after the discovery of penicillin in 1928, in 1940, several years before the introduction of penicillin to clinical practice, a bacterial penicillinase was identified by two members of the penicillin discovery team which suggested that antibiotic resistance could be intrinsic to the pathogen and not only acquired by the continuous exposure to a specific antibiotic (7). Most antimicrobial compounds are naturally produced molecules, and, therefore, coresident bacteria have evolved mechanisms to overcome their action to survive. It is only natural that organisms that produce antibiotics should also contain self-resistance mechanisms against their own antibiotics (8). Intrinsic resistance is the type of resistance that is naturally present in the

microorganism usually located to the chromosome and related to the general physiology of the organism. Therefore, intrinsic resistance happens due to the lack or presence of specific structures resulting in the ineffectiveness of antibiotics. For example, it is known that *Klebsiella penumoniae* has intrinsic resistance to ampicillin because it naturally produces a chromosomally encoded beta-lactamase that inactivate this molecule by cleaving the beta-lactam ring (9).

Once the antibiotic becomes widely deployed into clinical practice, it acts as a selective pressure driving the emergence of resistant strains that are capable of rendering the drug inactive or are impervious to its mode of activity. This often leads to treatment failure and also results in an increasing prevalence of the resistant strains in a given setting (10). This refers to a different type of resistance that is known as acquired resistance and can be achieved through different mechanisms such as mutations in chromosomal genes or via horizontal gene transfer (HGT) of chromosomes or plasmids (11). During DNA replication mutations can emerge as a result of replication and error correction errors and some of these can affect the activity of the drug leading to the emergence of a newly resistant strain that is able to outcompete the parental susceptible strain. This population will then replicate, and the mutation will become fixed, creating a resistant bacterial population. However, only few mutations can lead to drug resistance, these are usually mutations that occur in the gene coding for the drug target, transporters or that encode regulators that repress the expression of transporters (12) (13).

As well as preventing antibiotics from diffusing into the cell or altering their targets, bacteria can also destroy or modify antibiotics. Bacteria have developed several enzymatic hydrolysis mechanisms to inactivate such drugs. For example, beta-lactamases can hydrolyze the beta-lactam ring of penicillin. Some beta-lactamases are able to hydrolyze a broad range of beta-lactam antibiotics and are named extended spectrum beta-lactamases, or ESBLs. The TEM, SHV, and CTX-M ESBLs are now known to be produced by many gram-negative bacteria, including *Klebsiella pneumoniae*. The first plasmid harboring a beta-lactamase coding gene was described in Germany in 1983. At the same time, CTX-M enzymes were detected in humans and more recently in pets, farm animals, products from the food chain, and sewage (14). In the past decade, CTX-M enzymes have become the most prevalent extended spectrum beta-lactamases family of serine-beta-lactamases and represent a significant clinical concern due to the ability of these enzymes to confer resistance to a broad array of beta-lactam

antibiotics. The CTX-M-ase family was initially notable for its resistance to third-generation cephalosporins, cefotaxime, and ceftazidime while maintaining susceptibility to cefoxitin, latamoxef, or imipenem (15).

Another large family of beta-lactamase enzymes with hydrolytic activity are the carbapenemases that confer resistance to carbapenems. The KPC carbapenemases were first isolated from *Klebsiella pneumoniae* and quickly spread across a wide range of gram-negative bacteria and are no longer limited to *Klebsiella pneumoniae.* Carbapenems are frequently used for the treatment of bacterial infections as a last choice of therapy and therefore, the emergence and spread of these genes constitute a major public health problem across the healthcare setting as the therapeutic options remaining are more limited and less effective (16).

1.2. Emergence and Impact of Resistant Strains

Nowadays resistance is a major factor that has a big impact at different levels. It can lead to treatment failure and therefore have a negative impact on human health, and, at the same time, it has an economic impact. Moreover, resistance can compromise treatments that require immunosuppression or surgical procedures which require the use of antibiotic prophylaxis (17).

Some factors can promote antibiotic resistance such as the over prescription of broad spectrum antibiotics, paucity of diagnostic tests to guide not only adequate prescription but also guide the implementation of directed therapy, improper prescriptions, poor sanitation and contaminated water systems, global travel, antibiotic overuse in agriculture and in animal production, inadequate adherence to the treatment and bacterial population density in health care facilities (18).

As long as antibiotics are being used the selection pressure favor the emergence and persistence of resistant strains and its molecular determinants in the population or at a given setting. However, despite the increase in resistance, the number of new antibiotics has decreased by about 90% in the last 30 years. The global dissemination of resistance and this lack of development of new antibiotics is threatening to undo all the advances that the antibiotics have enabled, and it may lead to setback to a pre-antibiotic era or post-antibiotic one (19) (20).

With the discovery of laboratory methods to grow microorganisms using appropriate culture media, determining the sensitivity and resistance of a specific pathogen to a wide range of antimicrobial agents became possible, and necessary, so that healthcare providers can institute effective and directed treatment regimens. Antimicrobial susceptibility testing is used to identify which antimicrobial regimen is specifically effective for an individual patient. Antimicrobial susceptibility testing includes several methods such as disk diffusion and minimum inhibitory concentration (MIC). This susceptibility tests allows a microorganism to be determined as susceptible, intermediate, or resistant to the agent in question recurring to clinical breakpoints (21) (22).

Many bacterial pathogens have evolved into multidrug-resistant (MDR) forms especially due to the major use of antibiotics worldwide. The term "superbugs" refers to microorganisms with enhanced morbidity and mortality due to multiple mutations or resistance genes endowing high levels of resistance to the antibiotic classes specifically recommended for their treatment, the therapeutic options for these microorganisms are reduced, and periods of hospital care are extended, and more costly. In some cases, super-resistant strains have also acquired increased virulence and enhanced transmissibility (10). MDR is defined as non-susceptible to at least one agent in three or more antimicrobial classes. There are still XDR (extensively drug resistant) strains that are non-susceptible to at least one agent in all but two or fewer antimicrobial categories (23).

1.3. The ESKAPE Pathogens and *Klebsiella pneumoniae*

Klebsiella pneunoniae is a gram-negative rod-shaped bacteria that belongs to a group called ESKAPE. Antimicrobial resistant ESKAPE bacteria (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species*) show fewer treatment options due to their increasing association with antimicrobial resistance (24). This group of bacteria is often associated with nosocomial infections and *Klebsiella pneumoniae* is mainly responsible for urinary, respiratory, and bloodstream infections.

Cephalosporin and carbapenem antibiotic classes have been widely used for serious infections caused by Enterobacteriaceae, such as *Klebsiella pneumoniae*, but efficacy has been

compromised by the widespread acquisition of beta-lactamase coding genes (*bla*) such as extended spectrum beta-lactamases (ESBLs) and carbapenemases (25). Carbapenem resistant *Klebsiella pneumoniae* are ranked among the recently published WHO list of antibiotic-resistant priority/critical pathogens for which strengthened research is required (26).

For *Klebsiella pneumoniae* strains that seem resistant to these classes of antibiotics, other classes (such as aminoglycosides, polymyxins, and tigecycline) have been used as alternatives in combined therapy but with inherent toxicity problems and safety issues (27).

1.4. Antimicrobial Drugs

The definition of the term antibiotic was first described in 1947 as a chemical compound, produced by microorganisms with the capacity to inhibit or destroy other microbes. However, nowadays, this definition is no longer correct. Today an antibiotic is usually described as an antimicrobial substance, independent of whether it is of natural, semisynthetic, or synthetic origin, and, based on its structure and mode of action, at least seven major groups of antibiotics have been described which include beta-lactams (inhibit cell wall synthesis), aminoglycosides (protein synthesis), macrolides (protein synthesis), tetracyclines (protein synthesis), daptomycin (cell membrane function), platensimycin (fatty acid biosynthesis), glycopeptides (cell wall synthesis), among other classes (5). In this work, we focus on beta-lactam antibiotics and its resistance determinants.

1.4.1. Beta-lactam Drugs

The beta-lactam antibiotic group emerged with the discovery of penicillin, however, a gap of about ten years happened between the discovery of this compound and the large-scale production and therefore the clinical implementation of penicillin only occurred in 1941. Penicillin is characterized by having a beta-lactam ring that is the main molecular structure that exists between all beta-lactam antibiotics. Other groups include cephalosporins and carbapenems.

Regarding its mechanism of action, as mentioned above, beta-lactam antibiotics act by restricting the growth of the cell wall of bacteria, leading to restricting their growth and spread

and, eventually, cell death. The cell wall consists of a cross-link polymer of polysaccharides and polypeptides. The polysaccharides are formed via alternating amino sugar, N-acetyl glucosamine, and N-acetyl muramic acids. These terminate into D-alanyl-D-alanine structures. During bacterial growth, the penicillin-binding protein (PBP) removes the terminal alanine structure to form a cross-link with other peptides. As the name mentions, penicillin-binding proteins are the site of action of these antibiotics. The antibiotic binds to this receptor (PBP) and it is no longer able to remove the terminal alanine structure to make the cross-link across other peptides. This stops the production of the bacterial cell wall and leads to its consequent lysis (28).

1.4.1.1. Penicillins

Benzylpenicillin or penicillin G was the first natural produced antibiotic to be discovered and it was highly active and rapidly because it kills bacteria and does not merely prevent their growth. At the same time, penicillin has low toxicity, and it is a very cheap product, making it easier to be used in all patients. However, penicillin has some limitations such as the fact that it is not effective against all bacteria. Taking this into account, modifications to penicillin G structure were done in order to overcome some resistance barriers such as the acid lability of benzylpenicillin, its destruction by penicillinases, and its narrow spectrum of action. Therefore, other molecules were synthesized including penicillin V (resistant to the acid), methicillin, oxacillin, and cloxacillin (all resistant to penicillinases) and ampicillin, carbenicillin, and hetacillin (all with a large spectrum of action) (29).

1.4.1.2. Cephalosporins

The discovery of cephalosporins happened due to the isolation of a mold recovered from the sea near a sewage outfall. After being cultured, three potential antibiotics were isolated including cephalosporin C. Since then, a lot of studies have been done in order to make derivates with different characteristics that may enhance its activity. Thereby, different generations of cephalosporins were classified (28):

1.4.1.2.1. First-Generation

This generation has relatively narrow spectrum of activity focused mainly on the gram-positive bacteria including *Streptococci, Staphylococci,* and *Enterococci*, though susceptibilities may vary. This generation of cephalosporins is safer as they don't penetrate the cerebral spinal fluid.

Some examples include cefazolin (the most used in this group), cephalothin, and cephalexin (28).

1.4.1.2.2. Second-Generation

The second-generation of cephalosporins has a greater spectrum of activity against gram-negative bacteria with exception of anaerobes. These antibiotics are also more resistant to beta-lactamases and therefore are active against more bacteria such as *Hemophilus influenza, Moraxella catarrhalis, Proteus mirabilis, Escherichia coli, Klebsiella* and *Neisseria gonorrhea*. The most used antibiotic in this class is cefuroxime and cefoxitin which are used especially to treat lower respiratory tract infections, acute sinusitis, and otitis media (28).

1.4.1.2.3. Third-Generation

Third-generation cephalosporins are effective against gram-positive bacteria and gram-negative bacteria. They have bigger beta-lactamase stability and can penetrate the cell wall of gram-negative bacteria, killing them and preventing further infection. Cefotaxime, ceftriaxone, ceftazidime, ceftriaxone, ceftizoxime, and moxalactam are some of the most used third-generation cephalosporins.

In this work, only two third-generation cephalosporins were used in the assays performed: ceftazidime and cefotaxime. Ceftazidime is more active against PBP3 in gram-negative bacteria, such as *Pseudomonas aeruginosa*. Because of the emergence of new multiresistant strains and bla_{KCP}, bla_{CTX}, and bla_{SHV} genes that gives bacteria the ability to become resistant to this antibiotic, an association with avibactam has been used in clinical practices. Avibactam is a synthetic non-β-lactam beta-lactamase inhibitor active against beta-lactamases Ambler class A, B, and some D (30). However, there have been reports that mutations in the U-loop of the KPC gene (for example D179Y in KPC-3) can lead to a higher capacity of ceftazidime hydrolysis leading to resistance or low susceptibility (31) (32). The first clinical carbapenemase producing *Klebsiella pneumoni*ae in Portugal was isolated at a Lisbon hospital in 2009 (33). Since then, only sporadic infection isolates and single hospital cases have been reported, as well as a single outbreak of KPC-3 producing *Klebsiella pneumoniae* in 2013 (34).

Cefotaxime is a parenterally administered third-generation cephalosporin primarily used for the treatment of serious gram-negative bacterial infections. In general, cefotaxime is active against

Escherichia coli and species of *Proteus, Klebsiella, Enterobacter, Serratia, Providencia, Citrobacter, Shigella, Salmonella,* and *Yersinia*. However, the production of plasmid mediated extended spectrum beta-lactamases can also confer resistance among some Enterobacteriaceae (35) (36) (37).

1.4.1.2.4. Fourth-Generation

Fourth-generation cephalosporins have the broadest spectrum of activity with similar activity against gram-positive organisms as first-generation cephalosporins. They have a greater resistance to beta-lactamases than the third-generation cephalosporins. Cefepime and cefpirome are some examples of this group that are effective against gram-positive cocci, *Streptococcus, pneumoniae,* Enterobacteriaceae, and *Pseudomonas aeruginosa* (28).

1.4.1.2.5. Fifty-Generation

Ceftaroline is the only drug active against multidrug-resistant *Staphylococcus aureus*, including MRSA and it belongs to the fifty-generation of cephalosporins. Ceftobiprole is another fifty-generation cephalosporin with a very broad spectrum cephalosporin with activity against gram-positive cocci and many gram-negative bacilli (28).

1.4.1.3. Carbapenems

Overall, carbapenems demonstrated a broader antimicrobial spectrum than penicillin and cephalosporins (38). The first carbapenem ever discovered was thienamycin in the mid-1970s, a compound produced by the soil organism *Streptomyces cattleya*. Older carbapenems, such as imipenem, are often susceptible to degradation by the human enzyme dehydropeptidase-1 (DHP-1) and an inhibitor is usually administrated simultaneously in clinical settings (cilastatin). Meropenem and ertapenem have the advantage that no DHP-1 inhibitor is needed. In general, imipenem and panipenem are potent antibiotics against gram-positive bacteria and meropenem and ertapenem are slightly more effective against gram-negative organisms (39).

Its mechanism of action is by inhibiting the bacterial cell wall synthesis by binding to and inactivating penicillin-binding proteins. The binding of the beta-lactam molecule to the PBPs prevents bacteria from completing transpeptidation (cross-linking) of peptidoglycan strands. Imipenem binds preferentially to PBP2, followed by PBP1a and 1b, and has a weak affinity for

PBP3. Meropenem and ertapenem bind most strongly to PBP2, followed by PBP3, but also have strong affinities for PBP1a and PBP1b (40).

Carbapenem resistance in *Klebsiella pneumoniae* usually arises from 2 main mechanisms: permeability defects combined with overexpression of a beta-lactamase with weak carbapenemase activity (mostly CTX-M or AmpC cephalosporinases) and the acquisition of carbapenemases (41). Resistance to carbapenems develops when bacteria acquire or develop structural changes within their PBPs, when they acquire metallo-beta-lactamases that are capable of rapidly degrading carbapenems, or when changes in membrane permeability arise as a result of loss of specific outer membrane porins (39).

1.5. Objectives

The main objective of this work is to study the resistance levels to third-generation cephalosporins (ceftazidime and cefotaxime) and carbapenems (meropenem, imipenem and ertapenem) by *Klebsiella penumoniae* and correlate this results with their genetic background and molecular determinants, including different sequence types and resistance genes. Also, two different methods to study resistance levels were implemented with the aim to compare their results and their reliability.

The evolution of resistance was also the purpose of this study, and that is the reason the clinical isolates, used in the assays, were collected in different dates with the range of 39 years. This allows us to understand the evolution in resistance gene transfer along these years and presume what is most likely to happen in the following ones.

2. Material and Methods

2.1. Clinical Isolates

This study includes a total of 106 clinical isolates of *Klebsiella pneumoniae* available from the clinical isolate biobank of the Bacterial Pathogenomics and Drug Resistance Laboratory of the Research Institute for Medicines (iMed.Ulisboa), at the Faculty of Pharmacy of the University of Lisbon and were obtained from patients or medical instruments such as catheters or prothesis sources from different Hospitals in Portugal (herein designated as Hospital A to Hospital I) (Annex 1). The clinical isolates were obtained between 1980 and 2019, were kept stored by ultrafreezing, and regrown whenever necessary in Drigalski Lactose Agar at 37ºC overnight.

2.2. Drug Susceptibility Testing

Antimicrobial susceptibility testing was performed via disk diffusion assay on Muller Hinton agar and the determination of minimum inhibitory concentrations by broth microdilution. All assays were carried out as per the European Committee for Antimicrobial Susceptibility Testing (EUCAST) recommendations and standardized procedures and the interpretation of MICs and inhibitory zones were made according to EUCAST clinical breakpoints (42).

2.2.1. Kirby-Bauer Disk Diffusion

Disk diffusion based drug susceptibility testing was carried out for the antimicrobial drugs studied as per the recommendations of the EUCAST (42). Briefly, Muller-Hinton Agar plates were inoculated with a McFarland 0.5 standard suspension of the clinical isolate by streaking using a wood-shafted cotton swab in three different directions. Commercial antimicrobial containing disks (Ceftazidime 10 µg, Bio-rad Laboratories; Cefotaxime 5 µg, Bio-rad Laboratories; Meropenem 10 µg, Bio-rad Laboratories; Imipenem 10 µg, Bio-rad Laboratories; Ertapenem 10 µg, Bio-rad Laboratories) were applied at the surface of the agar plates and the plates incubated for 18 hours at 37ºC, as presented in Table 1. The diameter of the inhibition zones was recorded and compared with EUCAST clinical breakpoints (Table 1) (43).

2.2.2. Broth Microdilution

Broth microdilution (BMD) was carried out using untreated polystyrene 96 well plates (U-shaped, Greiner Bio-One, Frickenhausen, Germany) where the antibiotic concentrations were obtained by a serial two-fold dilution on Cation supplemented Muller Hinton Broth (Liofilchem Laboratories, Frilabo, Italy). The plates were prepared by adding 50 µL of drug free medium to all the wells but those in the last column to which 100 µL of the drug supplemented medium (at twofold the desired concentration) was added, followed by serial two-fold dilutions.

To prepare the inoculum, bacterial suspensions adjusted to a MacFarland 0.5 standard were prepared in sterile distilled water from overnight cultures in Muller Hinton agar (MHA). A 100-fold dilution was then prepared by adding 100 µL to 9.9 mL of Cation adjusted Muller Hinton Broth (Liofilchem Laboratories, Frilabo, Italy). Each well was subsequently inoculated with 50 µL of the inoculum, therefore reaching a final inoculum density of $5x10^5$ CFU/mL. Each assay included a medium sterility control (non-inoculated, negative control) and drug-free positive control (Figure 1). The plates were incubated overnight at 37ºC, and the results were observed on the following day. The minimum inhibitory concentration for each drug was defined as the lowest concentration that resulted in complete inhibition of visual growth. Interpretation and classification between resistance and susceptible were done according to EUCAST breakpoints for Enterobacteriaceae (44).

Antibiotic Stock Solutions were prepared for meropenem, ceftazidime, and cefotaxime at different concentrations according to the concentrations required (Table 1). All solutions were prepared by adding sterile water to the weighted powder of each antibiotic and then filtered with a 0.2 µg filter in a sterile environment. Antibiotic stock solutions were then aliquoted and frozen at -20ºC.

For quality control purposes two reference strains were used as controls: *Escherichia coli* ATCC 25922 (MIC_{MER}= 0.008-0.006 µg/mL; MIC_{CTA}= 0.03-0.012 µg/mL; MIC_{CTZ}= 0.06-0.5 µg/mL) and *Pseudomonas Aeruginosa* ATCC 27853 (MIC_{MER}= 0.12-1 µg/mL; MIC_{CTA}= 8-32 µg/mL; MIC_{CTZ}= 1-4 µg/mL) (45). The assays were considered valid if no growth was observed in the negative controls along with growth on positive control wells and, if the MICs obtained for the control strains were within the expected range.

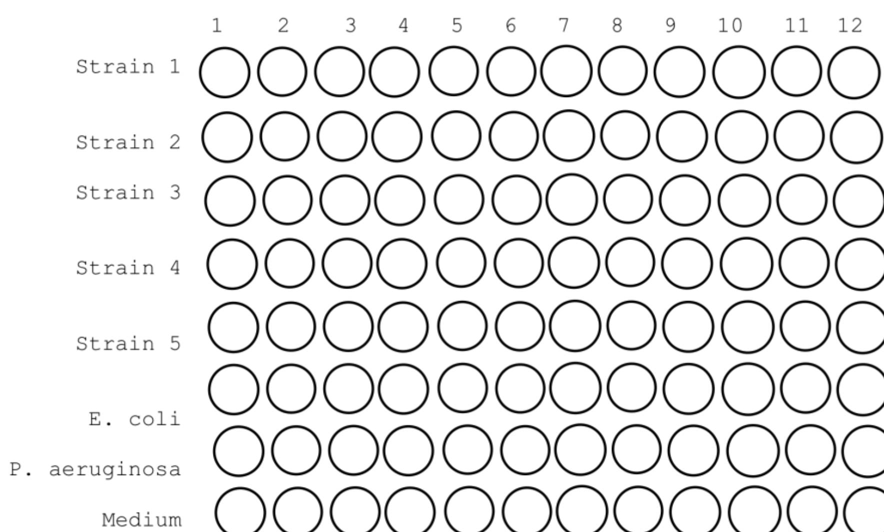


Figure 1 – Untreated polystyrene 96 well plates layout.

Representation of the 96 well plates. The first five rows correspond to five different strains to be tested and the next two concern the control strains. In the last row, there is a medium control to make sure that the medium was not contaminated. The first column works as a negative control and therefore is a free drug column where bacterial growth is expected. The next eleven columns have two-fold increased concentrations of the antibiotic tested.

Table 1 – Kirby-Bauer Disk Diffusion and MICs breakpoints.

Summary of the disk load of each antibiotic used in the disk diffusion assay and the concentration range in the broth microdilution method, as well as the stock solution concentrations prepared previously to be used in the assay.
Summary of the clinical breakpoints for Kirby Bauer disk diffusion assay and broth microdilution method to determine if the strain is susceptible, intermediate, or resistant to each antibiotic tested (ceftazidime, cefotaxime, meropenem, imipenem, and ertapenem) (43).

Drug	Kirby-Bauer Disk Diffusion				BMD/MIC Determination				
	Disk content (µg)	Zone diameter breakpoints (mm)			Stock Solution (µg/mL)	Concentration Range (µg/mL)	MIC breakpoints (µg/mL)		
		R	I	S			R	I	S
CTZ	10	< 19	19-21	≥ 22	1064	0.06 - 64	> 4	2-4	≤ 1
CTA	5	< 20		≥ 20	512	0.06 - 64	> 1		≤ 1
MER	10	< 22		≥ 22	1064	0.03 – 32	> 2		≤ 2
IMI	10	< 19	19-21	≥ 22					
ERT	10	< 25		≥ 25					

2.3. Bioinformatic Screening of Drug Resistance Genes and in silico MLST

All isolates included in the study had been previously subjected to whole genome sequencing via an Illumina sequencing platforming paired-end mode. Additionally, de novo assembly, which enables contig assembly without requiring a reference genome was carried out with the Unicycler which implements SPAdes assembler allowing for parametric optimization and also performs graph trimming removing overlaps between contigs.

Detection of drug resistance genes was subsequently performed from de novo assembled contigs using AMRFinder and the NCBI Bacterial Antimicrobial Resistance Reference Gene Database (Accession PRJNA313047) using a 60% coverage and 95% identity thresholds.

Sequence Type (ST) was determined by examining the seven housekeeping genes *rpoB, gapA, mdh, pgi, phoE, infB* and *tonB* in Institute Pasteur's MLST Web site (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

2.4. Statistical Analysis

Parameters including sensitivity (number of resistant strains that were correctly classified by the disk diffusion assay) and specificity (number of clinical isolates that were correctly classified as susceptible by disk diffusion) of each assay were calculated, as well as, three performance indices: categorical agreement (CA- the percentage of isolates with a concordant susceptible/resistant phenotype between the two methods), major error (ME- the percentage of false-resistant in the total susceptible isolates), and very major error (VME- the percentage of false-susceptible in the total resistant isolates). The results are considered within the acceptance criteria when CA $\geq 90\%$, ME $< 3\%$, and VME $< 3\%$ (45) (46).

3. Results and Discussion

This study includes a total of 106 clinical isolates of *Klebsiella pneumoniae* all of which tested for susceptibility to MER, CTZ, and CTA using Kirby-Bauer based disk diffusion assays on Muller Hinton agar and MIC determination by BMD (Annex 2, 3 and 4). In addition, all isolates were obtained from hospitals in Portugal and subjected to whole-genome sequencing as part of a large national genomic surveillance study carried out at the Research Institute for Medicines (iMed) and Faculty of Pharmacy of the University of Lisbon.

3.1. Classification of clinical isolates according to BMD

For MER, the MICs obtained ranged between ≤0.03 to ≥32.0 µg/mL with 83 (78.3%) classed as susceptible (MIC ≤2 µg/mL) whereas 23 (21.7%) isolates were classed as resistant with a MIC >2 µg/mL. Regarding CTZ the MICs ranged from ≤0.06 to ≥64.0 µg/mL with 24 (22.6%) susceptible strains (MIC ≤1 µg/mL) and 75 (70.8%) strains were classed as resistant (MIC >4 µg/mL). Seven (6.6%) clinical isolates demonstrated values between 2 and 4 µg/mL and therefore were classed as having intermediate resistance to CTZ. For CTA the MICs ranged between ≤0.06 to ≥64 µg/mL with 25 classed as susceptible (23.6%) (MIC ≤1 µg/mL) and 81 as resistant (76.4%) (MIC >1 µg/mL) (Figure 2). MICs of quality control strains were in accordance with the established values for the BMD method.

MIC50, MIC90, and MIC99 are defined as the value at which 50%, 90%, and 99% of the isolates are inhibited, respectively. We next compared the MIC90 and MIC99 for each drug. These metrics enable the quantification of the lowest MIC encompassing 90% and 99% of the studied isolates, as presented in Table 2.

Table 2 – MIC50, MIC90 and MIC99 obtained for meropenem, ceftazidime and cefotaxime.

	MER (µg/mL)	**CTZ (µg/mL)**	**CTA (µg/mL)**
MIC50	0.125	≥64	≥64
MIC90	≥32	≥64	≥64
MIC99	≥32	≥64	≥64

These results are congruent with the skewed distribution of CTZ and CTA MICs towards higher resistant levels whereas MER MICs show a clear bimodal distribution with an increased

prevalence of susceptible isolates. These results are also in agreement with the MIC distribution for each antibiotic, correlating the clinical isolate MICs obtained in the assay with the number of isolates that presented that MIC value (Figure 2), as well as the classification between susceptible, intermediate, or resistant (Table 3).

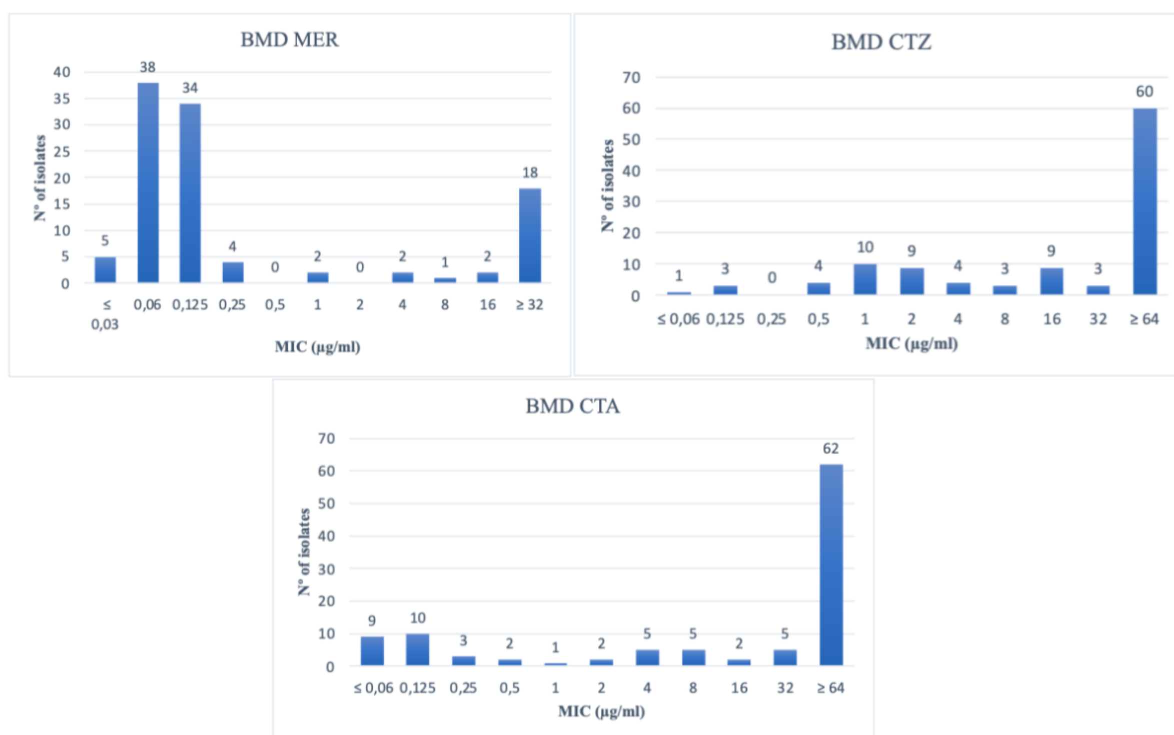


Figure 2 –MIC distribution obtained for meropenem, ceftazidime and cefotaxime.
Results obtained for Meropenem (MER), Ceftazidime (CTZ), and Cefotaxime (CTA) for susceptibility testing by BMD More clinical isolates presented higher MIC values to third-generation cephalosporins (CTZ and CTA) and the majority presented lower MIC values to MER.

Table 3 – Number of isolates according to categorical classification as determined by MIC determination.
Summary of how many clinical isolates presented as resistant, susceptible, or intermediate resistance to each antibiotic through broth microdilution assay.
More clinical isolates were classed as resistant to third-generation cephalosporins (CTZ and CTA) and the majority presented susceptibility to MER.

Drug	Categorial Classification			Total
	R	I	S	
MER	23	-	83	106
CTZ	75	7	24	106
CTA	81	-	25	106

3.2. Kirby-Bauer disk-diffusion based classification and distribution of inhibitory zone diameters

Regarding disk diffusion based susceptibility testing, 22 (20.8%) clinical isolates demonstrated susceptibility to CTZ (≥22 mm) and 82 (77.4%) resistance (<19 mm). The other two (1.9%) presented intermediate resistance (19-21 mm). Seventy-three (68.9%) strains were classed as phenotypically resistant to CTA (<20 mm) and 33 (31.1%) as susceptible (≥ 20 mm). A total of 84 (79.2%) clinical isolates would be classed as phenotypically susceptible to MER (≥22 mm) and 22 (20.8%) classed as resistant (<22 mm). Eighty-three (78.3%) clinical isolates were susceptible to IMI (≥22 mm) and 16 (15.1%) resistant (<19 mm). The other seven strains presented intermediate resistance (19-21 mm) (6.6%). A total of 25 (23.6%) clinical isolates were classified as phenotypically resistant to ERT (<25 mm) and 81 (76.4%) as susceptible (≥ 25 mm) (Figure 3).

Figure 3 – Distribution of IZ diameters.

Graphic correlation between the inhibition zone and the number of clinical isolates with that IZ value for Meropenem (MER), Ceftazidime (CTZ), Cefotaxime (CTA), Imipenem (IMI), and Ertapenem (ERT).

More clinical isolates presented smaller diameters for third-generation cephalosporins (CTZ and CTA) and higher values for MER, IMI, and ERT.

Examining and comparing the distribution of zone inhibition diameters (Figure 3), it is possible to observe similarities between CTZ and CTA and between MER, IMI, and ERT. For CTZ and CTA, lower inhibition zones diameters are across an increasing fraction of the study sample (demonstrating the increased prevalence of resistant strains) (Table 4). As for carbapenems, left-skewed distribution of inhibition zone diameters, suggests that the study sample is composed mainly by susceptible clinical isolates (Table 4). These results are congruent with the MICs and distribution obtained by BMD.

Table 4 – Number of isolates according to categorical classification as determined by disk diffusion.

Summary of how many clinical isolates presented as resistant, susceptible, or intermediate resistance to each antibiotic through Disk Diffusion assay.

More clinical isolates showed to be resistant to third-generation cephalosporins (CTZ and CTA) and susceptible for carbapenems (MER, IMI, and ERT).

Drug	Categorial Classification			Total
	R	I	S	
CTZ	82	2	22	106
CTA	73	-	33	106
MER	22	-	84	106
IMI	16	7	83	106
ERT	25	-	81	106

3.3. Distribution and Correlation of MICs and Inhibition Zones

Next, correlations between the minimum inhibitory concentration with the inhibitory zones were made for MER, CTZ, and CTA (Figure 4 and Annex 5). Also, correlations between minimum inhibitory zones of meropenem with inhibitory zones of IMI and ERT were made. Most clinical isolates presented a clear correlation between the two methods (Figure 4) showing that most clinical isolates are classed as susceptible through both methods for meropenem, imipenem and ertapenem. However, for some clinical isolates, no correlation seemed to be found as the results obtained through both methods are discordant. Especially in these clinical isolates, with different resistance levels, a better characterization should be performed along with replication of the assays to confirm these initial results.

CTZ — MIC (µg/ml) / Inhibition Zone (mm)	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
≤ 0.06									1																	
0.125						1		1					1													
0.25																										
0.5	1																					2		1		
1	1								1									1	1	1	1		4			
2	1																	1			1	3	2			1
4										1					1	1		1								
8								1			1									1						
16	2					1	1			1	1		2	1												
32										1		1	1													
> 64	28		7	4	1	4	4	2	4	4				1		1										

CTA — MIC (µg/ml) / Inhibition Zone (mm)	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
≤ 0.06											1								1		2		2	3				
0.125	1																			1	1			3	2	1		1
0.25	1						1																		1			
0.5																							1	1				
1																				1								
2																1			1									
4											1			1	1			1		1								
8																2	2		1									
16	1												1				1											
32						1				2		1																
> 64	31	4	4	6	3	4	3	3		1		2													1			

MER — MIC (µg/ml) / Inhibition Zone (mm)	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
≤ 0.03										1				1						1			1		1				
0.06									1						1				1	2	2	7	6	4	8	4	1	1	
0.125										1							1			2	2	3	8	4	4	7	1		1
0.25																			1			1	1		1				
0.5																													
1																		1						1					
2																													
4																1				1									
8	1																												
16									1					1															
> 32	2				2	2		1	2	2		1	1						1		1	2	1						

IMI — MIC (µg/ml) / Inhibition Zone (mm)	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
≤ 0.03																1				1	1	1	1				1			
0.06															1	2		2	1	5	2	3	7	6	4		1	2		1
0.125																1			5	2	7	6	7	2	2	2				
0.25															1		1					2								
0.5																														
1													1										1							
2																														
4																		1							1					
8																1														
16				1	1																									
> 32	5		1		2	2	3										1			1	2		1							

ERT — MIC (µg/ml) / Inhibition Zone (mm)	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
≤ 0.03																		1				1		1	3										1
0.06																		2	1	1	3	4	1	2	8	6	4	2		1					1
0.125								1								1			1	2	2	4	5	3	6	5	1		1						
0.25																				1			1	1	1										
0.5																																			
1															1										1										
2																																			
4														1	1																				
8													1																						
16									1	1																									
> 32	4			1	1		2		2			2									1	3	1		1										

Figure 4 - Correlation observed between MIC and IZ diameters.

Correlation between the MIC and DD assays. The black lines represent the limits for susceptibility (susceptible and resistant bacteria) for Klebsiella pneumoniae phenotypes according to EUCAST breakpoints.

IMI and ERT inhibitory zones were compared with MIC values of MER BMD assay.

Moreover, and to quantify the performance of DD using MIC determination by BMD as the reference method, agreement indexes were calculated using all 106 clinical isolates included in the study for the three different antibiotics (Table 5).

Regarding meropenem, categorical agreement (CA) was slightly below the acceptable value of ≥90%, with a value of 89.6%. The major error (ME) was 6.0% and the very major error was 26.0%, both elevated. Sensitivity and specificity were also calculated. Of all isolates, 17 out of 23 resistant strains were correctly classified, resulting in a sensitivity of 73.9% and 79 out of 83 were correctly classified as susceptible strains, resulting in a specificity of 95.2%. As for the strains with false results, five (4.7%) strains showed to be susceptible to meropenem through the reference method while demonstrating resistance to the disk diffusion method (Kp684, Kp725, Kp748, Kp898, and Kp4387). Also, six (5.7%) false susceptible strains with resistance to meropenem through the reference method and susceptibility through the disk diffusion assay were observed (Kp1495, Kp1507, Kp1528, Kp1675, Kp1677, and Kp5506).
Regarding the other two carbapenems, imipenem and ertapenem, sensitivity and specificity were also calculated (Table 6). For imipenem, 15 out of 23 clinical isolates were correctly classed as resistant resulting in a sensitivity of 65.2% and 76 out of 83 clinical isolates were correctly classed as susceptible resulting in a specificity of 91.6%. Regarding ertapenem, 17 out of 23 strains were correctly classified as resistant with a sensitivity of 73.9% and 75 out of 83 were correctly classed as susceptible resulting in a specificity of 90.4%.
When comparing these three antibiotics, the disk diffusion assay had better results with meropenem, with a sensitivity and specificity with the higher percentages. However, sensitivity in ertapenem assays had similar results as in meropenem. Regarding imipenem, it demonstrated the lowest sensitivity of all three. As for specificity, imipenem demonstrated to be more specific than ertapenem but not more than meropenem.

Regarding ceftazidime categorical agreement (CA) was slightly below the acceptable criteria, with a value of 85.9%. The major error and the very major error presented values of 37.5% and 6.7%, respectively. Of all isolates, 71 out of 73 resistant strains were correctly classified, resulting in a sensitivity of 97.3% and 17 out of 24 were correctly classified as susceptible strains, resulting in a specificity of 70.8%. As for the strains with false results, seven (6.6%) strains showed to be susceptible to ceftazidime through the reference method while demonstrating resistance through the disk diffusion being, Kp1495, Kp1507, Kp1528, Kp1677,

Kp3462, Kp3826, and Kp3832 and two (1.9%) clinical isolates presented as resistant strains in the disk diffusion assay while presenting intermediate resistance to ceftazidime in the BMD (Kp4855 and Kp4980). Also, one false susceptible strain demonstrated to be resistant to ceftazidime through the reference method and susceptible through the disk diffusion assay, Kp2476 and three clinical isolates demonstrated to be susceptible through the BMD assay while presenting intermediate resistance through the disk diffusion assay (Kp2224, Kp2497 and Kp5510).

Finally, cefotaxime presented a categorical agreement (CA) of 84.9% (slightly below criteria) and a major error and a very major error of 16.0% and 14.8% (both out of the limit of <3%). Of all isolates, 69 out of 81 resistant strains were correctly classified, resulting in a sensitivity of 85.5% and 21 out of 25 were correctly classified as susceptible strains, resulting in a specificity of 84.0%. As for the strains with false results, four (3.8%) clinical isolates were susceptible to cefotaxime through the reference method while resistance by disk diffusion method being, Kp1365, Kp3462, Kp3832, and Kp4980. Also, 12 (11.3%) false susceptible strains demonstrated resistance to meropenem through the reference method and susceptibility through the disk diffusion assay (Kp684, Kp725, Kp748, Kp1003, Kp2334, Kp2476, Kp3323, Kp4292, Kp4297, Kp4378, Kp4387, and Kp4408).

Despite some divergences in the results obtained, both methods showed to be adequate and reliable to evaluate the bacterial resistance levels. We have to consider that only a minority of the clinical isolates were incorrectly classified by the disk diffusion assay when in comparison with the refence method. Also, random, and systematic errors occur which can lead to different results of the assays, and we have to take them into account.

Table 5 – Performance of BMD and DD for drug susceptibility testing of MER, CTZ and CTA and respective agreement indexes.
Quantifying the performance of Disk Diffusion VS MIC determination and categorical classification.
Calculation of agreement and error indexes: CA (Number of isolates with Disk Diffusion assay results within the same categorical interpretation as reference method / total isolate)); ME (Number of isolates that yielded false-resistant results / number of isolates susceptible by the reference method); VME (Number of isolates that yielded false-susceptible results / number of isolates resistant by the reference method).

Drug	Method	Total	S	I	R	CA	ME	VMA
MER	BMD	106	83	-	23	89.6%	6.0%	26.0%
	DD		84	-	22			
CTZ	BMD	106	24	7	75	85.9%	37.5%	6.7%
	DD		22	2	82			
CTA	BMD	106	25	-	81	84.9%	16.0%	14.8%
	DD		33	-	73			

Table 6 – Sensitivity and Specificity

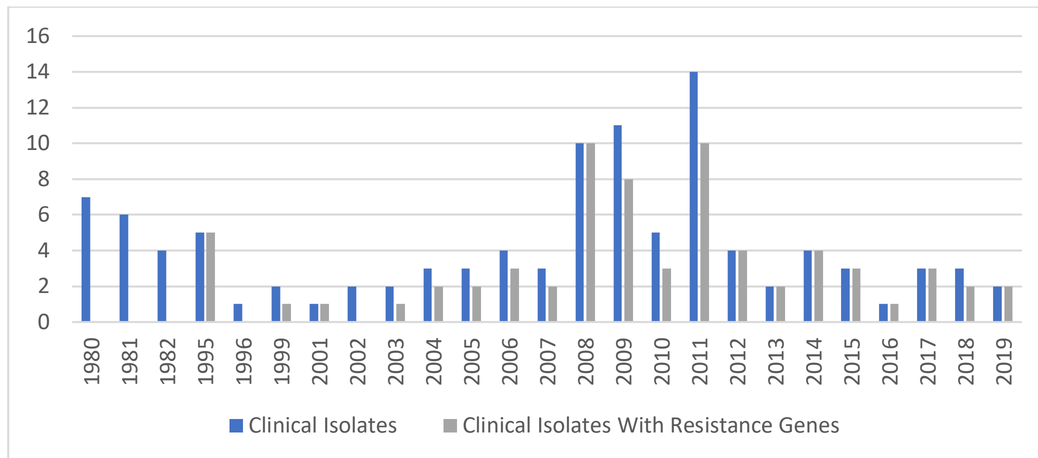
	Sensitivity	Specificity
MER	73.9%	95.2%
IMI	65.2%	91.6%
ERT	73.9%	90.4%
CTZ	97.3%	70.8%
CTA	85.5%	84.0%

3.4. Resistance Genes

In order to identify and correlate the molecular determinants of resistance with resistance levels, all isolates had been previously subjected to whole-genome sequencing and screened for drug resistance genes associated with resistance to third-generation cephalosporins and carbapenems. Among the 106 isolates, 53 (50.0%) bore ESBL coding genes, and 20 (18.9%) bore CARB coding genes (Annex 6). Four of these showed the concomitant presence of both ESBL and CARB genes (3.8%). Such resistance genes (coding for ESBL and CARB) were detected in isolates obtained between 1995 and 2019 with increasing prevalences observed towards more recent years (Figure 5). Although this is a diverse convenience sample composed of representative isolates from different STs and not sample with epidemiological representativeness, such increasing prevalences of ESBL and CARB producing isolates likely reflect the growing public health problem associated with drug resistance.

A total of 53 different STs were identified among the 106 clinical isolates. The most common sequence types among the clinical isolates were ST15 (12.3%), followed by ST13 (7.6%) and ST14, ST70, ST11, ST416, ST 147, and ST17 (Annex 1).

A)



B)

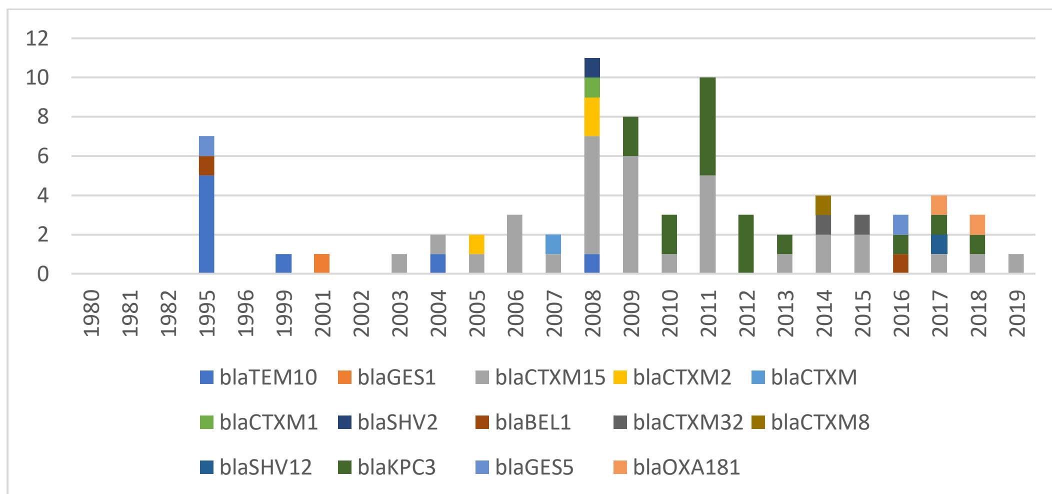


Figure 5 - Distribution of isolates carrying drug resistance genes (A) and selected beta-lactamase coding genes (B) per year. *In the first graphic, we can observe a comparison between the clinical isolates that have resistance genes and the ones that do not and the evolution and transmission of these resistance genes throughout the years.*
Graphic representation of all resistance genes in each strain categorized by the year of isolation.

Briefly, the most prevalent ESBL coding genes detected were $bla_{\text{CTX-M-15}}$ in 33 (31.1%) isolates and $bla_{\text{TEM-10}}$ in eight (7.6%) isolates (Table 7). Concerning CARB coding genes, a total of 20 (18.9%) isolates were found to bear at least one CARB coding gene. Across all isolates, 18 bore $bla_{\text{KPC-3}}$ (17.0%), two had $bla_{\text{GES-5}}$ (1.9%) and two $bla_{\text{OXA-181}}$ (1.9%). Overall, the most common resistance genes identified were $bla_{\text{CTX-M-15}}$, followed by $bla_{\text{KPC-3}}$ and $bla_{\text{TEM-10}}$. Noteworthy, a total of five clinical isolates co-harbored more than one resistant gene (n=5) ($bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-}}$

M-2); (*bla*BEL-1, *bla*TEM-10, *bla*GES-5); (*bla*GES-5, *bla*KPC-3, *bla*BEL-1) and two (*bla*OXA-181, *bla*CTX-M-15).

Among all the clinical isolates that presented the *bla*CTX-M-15 gene, only one (3.0%) showed to be susceptible to cefotaxime and the other 32 (97.0%) demonstrated resistance. Regarding ceftazidime, 29 isolates were considered resistant (17.2%), two were susceptible (6.1%) and two (6.1%) were classified with intermediate resistance. Regarding meropenem, the majority of the clinical isolates were susceptible to it. When in comparison with the mean MICs calculated in Table 7, we can confirm the results described above, with higher values of minimum inhibitory concentrations for cefotaxime, followed by ceftazidime, and that the MIC values for meropenem are much lower, indicating susceptibility to this antibiotic. The results obtained are therefore congruent with the predominant cefotaximase activity that is inherent to CTX-M enzymes and the absence of carbapenemase activity (14).

Among the clinical isolates harboring the *bla*KPC-3 gene, 14 (77.8%) strains were considered to be resistant to meropenem and only four (22.2%) were classed as susceptible. Regarding ceftazidime, 16 (88.9%) strains were resistant and only two (11.1%) clinical isolates were considered susceptible. As for cefotaxime, only one clinical isolate was considered susceptible to this antibiotic (5.6%) and all the other 17 strains showed to be resistant (94.4%). When in comparison with the mean MICs calculated in Table 7, we can confirm the results described above, with higher values of minimum inhibitory concentrations for cefotaxime, followed by ceftazidime.

Regarding the gene *bla*TEM-10, all clinical isolates harboring this gene showed resistance to ceftazidime, as well as for cefotaxime meanwhile all strains were susceptible to meropenem. When in comparison with the mean MICs calculated in Table 7, we can observe higher values of minimum inhibitory concentrations for ceftazidime, as it is expected, followed by cefotaxime and the MIC values for meropenem are much lower, indicating susceptibility to this antibiotic. The results obtained are therefore congruent with the predominant ceftazidimase activity that is inherent to TEM-10 enzymes and the absence of carbapenemase activity (47).
For the isolates harboring the gene *bla*CTX-M-2, one strain exhibited phenotypic resistance and the other two strains were susceptible to meropenem. Regarding ceftazidime, two clinical isolates were resistant and two was susceptible. As for cefotaxime, all three clinical isolates

demonstrated to be resistant. When in comparison with the mean MICs calculated in Table 7, cefotaxime has the higher MIC, followed by ceftazidime and meropenem, as in agreement with the results described above.

Table 7 – Frequency and mean MICs associated with beta-lactamase coding genes.
Correlation between all the resistant genes and the mean minimum inhibitory concentration of all the isolates harboring each gene. Also, the range of minimum inhibitory concentration for each gene making it easier to understand the variation between all the clinical isolates with a determinate gene.

Resistance Gene	Frequence	Mean MIC (µg/mL)		
		MER (range)	CTZ (range)	CTA (range)
*bla*TEM-10	8	0.105 (0.03-0.125)	58 (16-64)	13.75 (2-16)
*bla*GES-1	1	0.06	64	8
*bla*CTX-M-15	33	3.14 (0.06-32)	46 (0.06-64)	62.07 (0.25-64)
*bla*CTX-M-2	3	10.77 (0.06-32)	32.12 (0.125-16)	64
*bla*CTX-M	1	32	0.125	64
*bla*CTX-M-1	1	32	0.125	64
*bla*CTX-M-8	1	0.06	4	64
*bla*SHV-2	1	0.06	4	4
*bla*BEL-1	2	16.06 (0.125-32)	64	33 (2-64)
*bla*CTX-M-32	2	2.06 (0.125-4)	64	64
*bla*SHV-12	1	0.125	64	64
*bla*KPC-3	18	20.9 (0.125-32)	57 (1-64)	58.68 (0.25-64)
*bla*GES-5	2	16.06 (0.125-32)	64	33 (2-64)
*bla*OXA-181	2	2.5 (1-4)	64	64

Concerning the *bla*OXA-181 gene, reports have shown that it has emerged among clinical isolates in Portugal at an increased rate over time in hospitals since 2016 (likely mediated by plasmid lateral transfer). The finding of this gene in clinical isolates obtained between 2017 and 2018 is congruent with those previous reports. Additionally, some studies report that some OXA-181

producing isolates remained susceptible to imipenem and meropenem (34). Concerning the clinical isolates herein studied, two presented the *bla*$_{OXA-181}$ gene, all from the same sequence type (ST17) and co-harboring the *bla*$_{CTX-M-15}$ gene. Both strains were resistant to ceftazidime and cefotaxime, susceptible to meropenem by the disk diffusion assay and the Kp 5506 exhibited susceptibility to imipenem whereas Kp5511 was resistant. Nonetheless, both showed to be resistant to ertapenem. These findings are further corroborated by the lower mean MER MIC associated with OXA-181 producing isolates when comparing with KPC-3 producing isolates (2.5 vs 20.9, respectively; Table 7).

This study includes one clinical isolate that co-harbored the genes *bla*$_{KPC-3}$ and *bla*$_{GES-5}$. The gene *bla*$_{GES-5}$ has been found previously in environmental sources and in one clinical isolate in Portugal but never in combination with *bla*$_{KPC-3}$ (48). This raises further concern regarding resistance dissemination and clinical impact, as these strains showed a higher MIC value to cefotaxime. This data obtained by BMD and DD lends further support to this notion since the isolate was deemed resistant to all antibiotics.

Both the *bla*$_{BEL-1}$ and the *bla*$_{GES-5}$ genes have been identified in *Pseudomonas aeruginosa*, associated with a ColE1 plasmid suggesting that these genes may originate from *Pseudomonas aeruginosa*. Co-producers of GES-5 and BEL-1 enzymes showed decreased susceptibility to imipenem, meropenem, and ertapenem (34). Our results indicate that one strain Kp 4887 is resistant to all carbapenems tested, however, the other strain Kp 4297 is susceptible to all, therefore there is no apparent correlation.

As mentioned before, the CTX-M-ase family was initially notable for its resistance to the third-generation cephalosporins while maintaining susceptibility to imipenem (15). We can observe that the 2 strains caring the *bla*$_{CTX-M-32}$ gene (Kp 4859 and Kp 4864) have resistance to cefotaxime and ceftazidime. Regarding carbapenems, the strain Kp 4864, demonstrate to be susceptible to only imipenem and the Kp 4859 is susceptible to all carbapenems tested. Regarding other *bla*$_{CTX-M}$ family genes (including *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M}$, and *bla*$_{CTX-M-8}$) they all presented similar results, demonstrating susceptibility to carbapenems and resistance to the third-generation cephalosporin antibiotics.

Only one clinical isolate had the gene *bla*$_{GES-1}$ in its genome, conferring resistance to cephalosporins but not to carbapenems. All the other strains that presented ESBL genes (*bla*$_{TEM-10}$, *bla*$_{SHV-2}$, and *bla*$_{SHV-12}$) showed similar results among them.

4. Conclusion

Considering the increasing identification of carbapenemase and beta-lactamase producing *Klebsiella pneumoniae* in hospitals, systematic carriage screening at hospital admission, additional surveillance studies, and early detection of such isolates are required to limit their further spread. These measures would help mitigate the spread of these isolates in Portugal.

Studies regarding the discovery of new antibiotics for, not only *Klebsiella pneumoniae* but also for all bacteria presented in the ESKAPE group is in urgent need, due to the easy and rapid transmissibility of resistance genes among different clinical isolates and different bacteria of the same family.

This work provides further data on the resistance levels to carbapenems and third-generation cephalosporins in *Klebsiella pneumoniae* over time in Portugal, including isolates from 1980 to 2019. The study has some limitations since the clinical isolates were collected from different hospitals in the region of Lisbon and are not representative at a country-wide level and the fact that the sample is enriched for drug resistant strains. In conclusion, this study provides further data on the resistance levels to third-generation cephalosporins and carbapenems while correlating with molecular determinants. Most clinical isolates were resistant to third-generation cephalosporins (CTZ and CTA) but susceptible to carbapenems (MER, IMI and ERT) owing to a clear predominance of *Klebsiella pneumoniae* CTX-M-15 and KPC-3 producing strains. Moreover, relevant data on the correlation and sensitivity of DD method for the detection of drug resistance is herein reported but further studies should be performed to confirm the results obtained, especially for the clinical isolates that presented with different results among the two different AST performed.

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6. Annexes

Annex 1

106 clinical isolates were used in this study. In this table we can observe information regarding the hospital, source, and harvest date that the strains were collected. All the clinical isolates were kept stored in the facilities of the Faculty of Pharmacy of the University of Lisbon by ultrafreezing.

Strain	Species	Sequece Type	Hospital	Source	Harvest Date
684	*Klebsiella pneumoniae*	ST25	H-A	Urine	08/02/99
725	*Klebsiella pneumoniae*	ST12	H-A	Blood	28/10/99
748	*Klebsiella pneumoniae*	ST14	H-A	Blood	04/01/01
804	*Klebsiella pneumoniae*	ST45-2LV	H-A	Blood	06/09/02
809	*Klebsiella pneumoniae*	ST12	H-A	Blood	07/10/02
840	*Klebsiella pneumoniae*	ST43	H-A	Blood	09/05/03
888	*Klebsiella pneumoniae*	ST15	H-A	Blood	18/11/03
898	*Klebsiella pneumoniae*	ST45	H-A	Blood	06/01/04
997	*Klebsiella pneumoniae*	ST15	H-A	Blood	13/11/04
1003	*Klebsiella pneumoniae*	ST15	H-A	Blood	06/12/04
1031	*Klebsiella pneumoniae*	ST15	H-A	Blood	16/03/05
1094	*Klebsiella pneumoniae*	ST192	H-A	Blood	15/09/05
1144	*Klebsiella pneumoniae*	ST15	H-A	Blood	03/04/06
1209	*Klebsiella pneumoniae*	ST35	H-A	Blood	15/04/06
1264	*Klebsiella pneumoniae*	ST14	H-A	Blood	27/07/07
1365	*Klebsiella pneumoniae*	ST76	H-B	Blood	27/11/05
1495	*Klebsiella pneumoniae*	ST147	H-A	Blood	01/12/07
1507	*Klebsiella pneumoniae*	ST133	H-A	Blood	17/12/07
1528	*Klebsiella pneumoniae*	ST39	H-A	Blood	08/01/08
1675	*Klebsiella pneumoniae*	ST48	H-B	Blood	04/04/08
1677	*Klebsiella pneumoniae*	ST1037	H-C	Urine	20/03/08
1788	*Klebsiella pneumoniae*	ST1037	H-C	Feces	19/03/08
1990	*Klebsiella pneumoniae*	ST29	H-A	Blood	08/04/08
2058	*Klebsiella pneumoniae*	ST13	H-C	Catheter	17/06/06
2063	*Klebsiella pneumoniae*	ST70	H-C	Catheter	13/09/05
2069	*Klebsiella pneumoniae*	ST70	H-C	Urine	15/02/06
2200	*Klebsiella pneumoniae*	ST336	H-A	Blood	04/08/08
2209	*Klebsiella pneumoniae*	ST133	H-A	Urina	11/08/08
2224	*Klebsiella pneumoniae*	ST2176	H-A	Blood	16/08/08
2334	*Klebsiella pneumoniae*	ST20	H-A	Blood	13/10/08

2447	*Klebsiella pneumoniae*	ST730	H-A	Blood	22/12/08
2463	*Klebsiella pneumoniae*	ST218	H-A	Blood	09/01/09
2476	*Klebsiella pneumoniae*	ST13	H-A	Blood	30/01/09
2497	*Klebsiella pneumoniae*	ST134	H-A	Blood	17/02/09
2564	*Klebsiella pneumoniae*	ST11	H-A	Blood	11/04/09
2568	*Klebsiella pneumoniae*	ST336	H-A	Blood	03/04/09
2587	*Klebsiella pneumoniae*	ST336	H-A	Blood	11/04/09
2645	*Klebsiella pneumoniae*	ST13	H-A	Blood	09/06/09
2741	*Klebsiella pneumoniae*	ST231	H-A	Blood	04/09/09
2786	*Klebsiella pneumoniae*	ST152	H-A	Blood	06/10/09
2864	*Klebsiella pneumoniae*	ST1801	H-A	Blood	27/11/09
2895	*Klebsiella pneumoniae*	ST726	H-A	Blood	23/12/09
2948	*Klebsiella pneumoniae*	ST14	H-A	Pus	05/02/10
3000	*Klebsiella pneumoniae*	ST231	H-A	Blood	19/02/10
3109	*Klebsiella pneumoniae*	ST76	H-A	Urine	27/06/10
3185	*Klebsiella pneumoniae*	ST15	H-A	Blood	08/08/10
3270	*Klebsiella pneumoniae*	ST348	H-A	Blood	13/01/11
3323	*Klebsiella pneumoniae*	ST11	H-A	Blood	16/02/11
3389	*Klebsiella pneumoniae*	ST11	H-A	Blood	21/03/11
3396	*Klebsiella pneumoniae*	ST39	H-A	Blood	26/03/11
3462	*Klebsiella pneumoniae*	ST416	H-A	Urine	02/05/11
3501	*Klebsiella pneumoniae*	ST416	H-A	Urine	27/05/11
3635	*Klebsiella pneumoniae*	ST15	H-A	Blood	26/08/11
3666	*Klebsiella pneumoniae*	ST11	H-A	Urine	20/09/11
3715	*Klebsiella pneumoniae*	ST405	H-A	Blood	28/10/11
3718	*Klebsiella pneumoniae*	ST187	H-A	Prothesis	27/10/11
3734	*Klebsiella pneumoniae*	ST416	H-A	Urine	22/11/11
3760	*Klebsiella pneumoniae*	ST416	H-A	Urine	19/10/10
3807	*Klebsiella pneumoniae*	ST960	H-A	Blood	26/05/12
3826	*Klebsiella pneumoniae*	ST1138	H-A	Urine	16/07/12
3832	*Klebsiella pneumoniae*	ST6004	H-A	Blood	16/08/12
3854	*Klebsiella pneumoniae*	ST147	H-A	Blood	23/11/12
4129	*Klebsiella pneumoniae*	ST14	H-A	Ear Swab	29/02/80
4171	*Klebsiella pneumoniae*	ST1728	H-A	Belly Button	10/03/81
4179	*Klebsiella quasipneumoniae*	ST3520	H-A	Pharyngeal Exudate	07/12/81
4184	*Klebsiella pneumoniae*	ST37	H-A	Pharyngeal Exudate	07/12/81
4197	*Klebsiella pneumoniae*	ST1799	H-A	Feces	01/02/82
4214	*Klebsiella pneumoniae*	ST2493	H-A	Bench Top	24/11/81
4228	*Klebsiella pneumoniae*	ST3833	H-D	Urine	09/08/80
4238	*Klebsiella pneumoniae*	ST20	H-D	SB	25/10/80
4246	*Klebsiella pneumoniae*	ST15	H-D	Urine	17/11/80

4248	*Klebsiella pneumoniae*	ST3	H-D	SB	17/11/80
4249	*Klebsiella pneumoniae*	ST36	H-D	SB	17/11/80
4257	*Klebsiella pneumoniae*	ST15	H-D	Urine	05/12/80
4263	*Klebsiella pneumoniae*	ST15	H-D	Urine	24/01/81
4265	*Klebsiella pneumoniae*	ST289-2LV	H-D	Urine	31/01/81
4275	*Klebsiella pneumoniae*	ST13	H-D	Feces	16/01/82
4277	*Klebsiella pneumoniae*	ST111	H-D	Urine	23/01/82
4279	*Klebsiella pneumoniae*	ST13	H-D	Catheter	01/02/82
4292	*Klebsiella pneumoniae*	ST70	H-A	Blood	09/04/95
4297	*Klebsiella pneumoniae*	ST25	H-A	Blood	02/05/95
4333	*Klebsiella pneumoniae*	ST252	H-A	Blood	26/06/95
4378	*Klebsiella pneumoniae*	ST147	H-A	Blood	16/10/95
4387	*Klebsiella pneumoniae*	ST158	H-A	Blood	09/11/95
4408	*Klebsiella pneumoniae*	ST25	H-A	Blood	18/01/96
4852	*Klebsiella pneumoniae*	ST960	H-E	Urine	24/07/13
4855	*Klebsiella pneumoniae*	ST37	H-E	SB	15/12/13
4859	*Klebsiella pneumoniae*	ST423	H-E	Urine	22/05/14
4860	*Klebsiella pneumoniae*	ST307	H-E	Rectal	16/09/14
4861	*Klebsiella pneumoniae*	ST17	H-E	Rectal	17/09/14
4862	*Klebsiella pneumoniae*	ST348	H-E	Catheter	26/10/14
4864	*Klebsiella pneumoniae*	ST35-1LV	H-E	Urine	21/01/15
4865	*Klebsiella pneumoniae*	ST70	H-E	Urine	17/03/15
4869	*Klebsiella pneumoniae*	ST15	H-E	Blood	05/01/11
4871	*Klebsiella pneumoniae*	ST307	H-E	Blood	27/05/15
4887	*Klebsiella pneumoniae*	ST147	H-E	Rectal	25/04/16
4939	*Klebsiella pneumoniae*	ST20	H-F	Expect	30/08/11
4958	*Klebsiella pneumoniae*	ST15	H-F	Urine	05/12/11
4980	*Klebsiella pneumoniae*	ST13	H-F	Umbilical Catheter	27/04/17
4982	*Klebsiella pneumoniae*	ST348	H-G	Catheter	09/07/17
4998	*Klebsiella pneumoniae*	ST147	H-H	Urine	2017
5506	*Klebsiella pneumoniae*	ST17	H-I	Urine	19/10/18
5509	*Klebsiella pneumoniae*	ST17	H-B	Urine	22/08/18
5510	*Klebsiella pneumoniae*	ST13	H-B	Blood	2018
5511	*Klebsiella pneumoniae*	ST17	H-D	Blood	02/03/19
5518	*Klebsiella pneumoniae*	ST13	H-I	Blood	12/05/19

Annex 2

Table with the results of each AST performed, as well as the classification of resistant, susceptible, or intermediate resistance to MER, IMI, and ERT.

MIC and DD MER columns is the result obtained in the broth microdilution and the disk diffusion assay. On the right side of each column there is the classification obtained according to EUCAST breakpoints as susceptible (S), resistant (R) or with intermediate resistance (_). Highlighted in green are the strains with similar resistance levels in the two methods preformed. DD IMI and DD ERT are the results for the disk diffusion assay for imipenem and ertapenem.

STRAIN	MIC MER	R/S	DD MER	R/S	DD IMI	R/S	DD ERT	R/S
684	≤0.03	S	15	R	26	S	27	S
725	≤0.03	S	19	R	28	S	29	S
748	0.06	S	20	R	30	S	30	S
804	≤0.03	S	28	S	21	_	30	S
809	≤0.03	S	25	S	25	S	30	S
840	≤0.03	S	30	S	27	S	23	R
888	0.06	S	27	S	29	S	27	S
898	0.06	S	14	R	28	S	26	S
997	0.06	S	24	S	30	S	30	S
1003	0.06	S	28	S	25	S	23	R
1031	0.06	S	30	S	23	S	27	S
1094	0.06	S	25	S	20	_	26	S
1144	0.06	S	27	S	23	S	30	S
1209	0.06	S	28	S	24	S	30	S
1264	0.25	S	27	S	22	S	29	S
1365	0.06	S	26	S	32	S	30	S
1495	>32	R	26	S	26	S	27	S
1507	>32	R	27	S	26	S	28	S
1528	32	R	24	S	22	S	26	S
1675	>32	R	28	S	28	S	27	S
1677	>32	R	27	S	25	S	27	S
1788	0.125	S	25	S	24	S	27	S
1990	0.25	S	24	S	20	_	28	S
2058	0.06	S	27	S	25	S	33	S
2063	0.125	S	22	S	28	S	28	S
2069	0.125	S	28	S	25	S	13	R
2200	0.06	S	27	S	27	S	30	S
2209	0.06	S	28	S	26	S	26	S
2224	0.06	S	30	S	25	S	29	S

2334	0.125	S	31	S	27	S	30	S
2447	0.125	S	28	S	26	S	31	S
2463	0.125	S	31	S	31	S	29	S
2476	0.06	S	28	S	30	S	29	S
2497	0.125	S	28	S	26	S	34	S
2564	>32	R	15	R	6	R	30	S
2568	0.125	S	25	S	24	S	29	S
2587	0.125	S	30	S	26	S	28	S
2645	0.125	S	29	S	26	S	27	S
2741	0.125	S	26	S	24	S	30	S
2786	0.125	S	27	S	30	S	30	S
2864	0.125	S	27	S	30	S	30	S
2895	8	R	6	R	21	_	13	R
2948	>32	R	10	R	11	R	14	R
3000	0.06	S	28	S	30	S	31	S
3109	0.06	S	31	S	33	S	32	S
3185	0.06	S	25	S	21	_	27	S
3270	0.125	S	30	S	21	_	32	S
3323	0.06	S	27	S	27	S	27	S
3389	>32	R	14	R	8	R	12	R
3396	0.06	S	27	S	21	_	30	S
3462	0.06	S	26	S	27	S	40	S
3501	16	R	19	R	9	R	15	R
3635	0.125	S	28	S	28	S	31	S
3666	>32	R	11	R	11	R	12	R
3715	0.125	S	29	S	27	S	28	S
3718	32	R	10	R	12	R	14	R
3734	32	R	14	R	12	R	10	R
3760	32	R	13	R	6	R	6	R
3807	32	R	17	R	12	R	17	R
3826	32	R	18	R	10	R	17	R
3832	0.06	S	33	S	35	S	40	S
3854	>32	R	6	R	6	R	6	R
4129	0.125	S	27	S	31	S	30	S
4171	0.06	S	30	S	33	S	35	S
4179	0.06	S	28	S	29	S	32	S
4184	0.06	S	29	S	28	S	31	S
4197	0.06	S	29	S	28	S	32	S
4214	0.06	S	31	S	28	S	28	S
4228	0.125	S	28	S	26	S	29	S
4238	0.06	S	29	S	28	S	31	S
4246	0.06	S	30	S	29	S	31	S

4248	0.06	S	29	S	28	S	30	S
4249	0.06	S	30	S	28	S	31	S
4257	0.06	S	30	S	29	S	31	S
4263	0.06	S	31	S	32	S	33	S
4265	0.06	S	30	S	29	S	30	S
4275	0.25	S	30	S	27	S	30	S
4277	0.125	S	31	S	29	S	31	S
4279	0.125	S	34	S	27	S	31	S
4292	0.125	S	31	S	28	S	27	S
4297	0.125	S	29	S	29	S	28	S
4333	0.125	S	31	S	24	S	26	S
4378	0.125	S	28	S	26	S	26	S
4387	0.125	S	15	R	26	S	25	S
4408	0.125	S	31	S	27	S	28	S
4852	>32	R	15	R	6	R	6	R
4855	0.06	S	31	S	25	S	25	S
4859	0.125	S	30	S	27	S	25	S
4860	0.125	S	32	S	28	S	26	S
4861	0.06	S	27	S	25	S	23	R
4862	0.06	S	32	S	26	S	24	R
4864	4	R	21	R	30	S	20	R
4865	0.125	S	30	S	28	S	24	R
4869	0.125	S	28	S	27	S	26	S
4871	0.125	S	26	S	24	S	21	R
4887	>32	R	6	R	6	R	6	R
4939	0.25	S	28	S	27	S	25	S
4958	1	S	29	S	28	S	30	S
4980	0.06	S	30	S	29	S	32	S
4982	>32	R	11	R	10	R	9	R
4998	0.125	S	31	S	28	S	30	S
5506	4	R	25	S	23	S	19	R
5509	16	R	14	R	10	R	14	R
5510	0.125	S	29	S	25	S	31	S
5511	1	S	23	S	18	R	20	R
5518	0.125	S	28	S	27	S	27	S

Annex 3

Table with the results of each AST performed, as well as the classification of resistant, susceptible, or intermediate resistance to CTZ.

MIC and DD CTZ columns is the result obtained in the broth microdilution and the disk diffusion assay. On the right side of each column there is the classification obtained according to EUCAST breakpoints as susceptible (S), resistant (R) or with intermediate resistance (_). Highlighted in green are the strains with similar resistance levels in the two methods preformed.

STRAIN	MIC CTZ	R/S	DD CTZ	R/S
684	>64	R	6	R
725	4	_	21	_
748	>64	R	6	R
804	>64	R	8	R
809	32	R	15	R
840	>64	R	6	R
888	>64	R	8	R
898	16	R	11	R
997	8	R	16	R
1003	>64	R	8	R
1031	>64	R	15	R
1094	16	R	16	R
1144	16	R	12	R
1209	32	R	17	R
1264	>64	R	14	R
1365	8	R	13	R
1495	0.125	S	11	R
1507	0.06	S	14	R
1528	0.125	S	18	R
1675	>64	R	9	R
1677	0.125	S	13	R
1788	>64	R	13	R
1990	16	R	18	R
2058	16	R	19	R
2063	>64	R	15	R
2069	>64	R	19	R
2200	>64	R	6	R
2209	>64	R	12	R
2224	4	_	23	S
2334	>64	R	8	R

2447	16	R	18	R
2463	>64	R	14	R
2476	8	R	25	S
2497	2	_	28	S
2564	>64	R	11	R
2568	>64	R	15	R
2587	64	R	14	R
2645	>64	R	6	R
2741	>64	R	6	R
2786	64	R	6	R
2864	16	R	6	R
2895	>64	R	9	R
2948	>64	R	6	R
3000	>64	R	6	R
3109	>64	R	14	R
3185	64	R	12	R
3270	16	R	6	R
3323	32	R	18	R
3389	>64	R	9	R
3396	64	R	11	R
3462	1	S	14	R
3501	>64	R	6	R
3635	>64	R	8	R
3666	>64	R	8	R
3715	>64	R	6	R
3718	>64	R	6	R
3734	>64	R	11	R
3760	>64	R	6	R
3807	>64	R	10	R
3826	1	S	6	R
3832	0.5	S	6	R
3854	>64	R	6	R
4129	0.5	S	29	S
4171	1	S	26	S
4179	1	S	24	S
4184	0.5	S	27	S
4197	2	S	27	S
4214	1	S	23	S
4228	2	S	28	S
4238	0.5	S	27	S
4246	1	S	28	S
4248	1	S	25	S

4249	2	S	26	S
4257	1	S	28	S
4263	2	S	27	S
4265	1	S	28	S
4275	1	S	28	S
4277	2	S	27	S
4279	2	S	31	S
4292	>64	R	6	R
4297	>64	R	8	R
4333	>64	R	6	R
4378	>64	R	6	R
4387	>64	R	6	R
4408	>64	R	6	R
4852	>64	R	6	R
4855	4	_	15	R
4859	>64	R	11	R
4860	>64	R	12	R
4861	4	_	20	_
4862	>64	R	13	R
4864	>64	R	15	R
4865	16	R	15	R
4869	>64	R	6	R
4871	>64	R	12	R
4887	>64	R	6	R
4939	>64	R	9	R
4958	64	R	21	_
4980	2	_	6	R
4982	>64	R	6	R
4998	>64	R	6	R
5506	>64	R	6	R
5509	>64	R	6	R
5510	2	_	23	S
5511	>64	R	6	R
5518	64	R	6	R

Annex 4

Table with the results of each AST performed, as well as the classification of resistant, susceptible, or intermediate resistance to CTA.

MIC and DD CTA columns is the result obtained in the broth microdilution and the disk diffusion assay. On the right side of each column there is the classification obtained according to EUCAST breakpoints as susceptible (S), resistant (R) or with intermediate resistance (_). Highlighted in green are the strains with similar resistance levels in the two methods preformed.

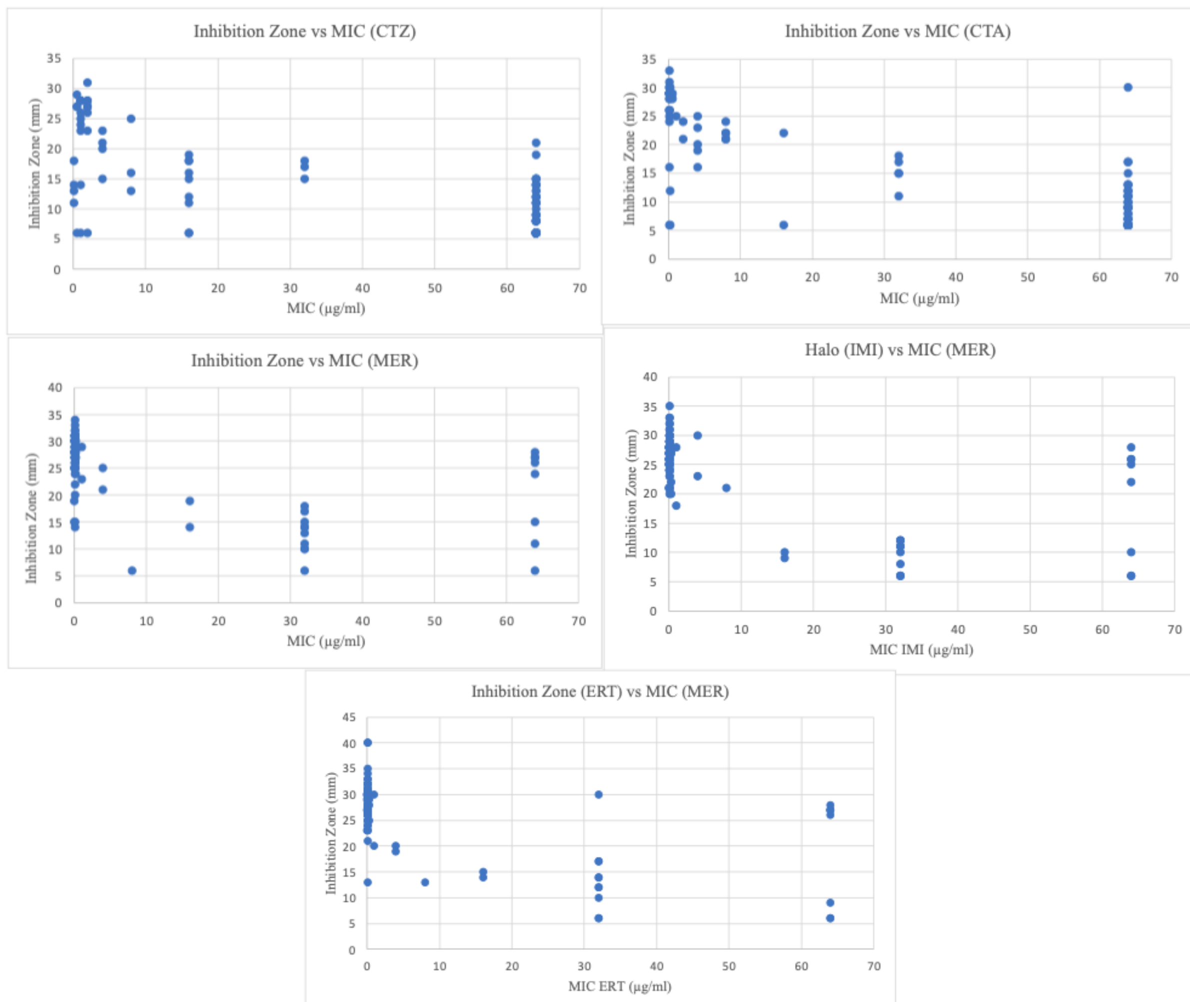
STRAIN	MIC CTA	R/S	DD CTA	R/S
684	8	R	24	S
725	2	R	21	S
748	8	R	22	S
804	>64	R	15	R
809	32	R	11	R
840	64	R	11	R
888	>64	R	6	R
898	>64	R	6	R
997	>64	R	9	R
1003	8	R	21	S
1031	>64	R	6	R
1094	>64	R	6	R
1144	>64	R	6	R
1209	0.125	S	25	S
1264	>64	R	9	R
1365	0.125	S	6	R
1495	>64	R	6	R
1507	>64	R	8	R
1528	>64	R	8	R
1675	>64	R	9	R
1677	>64	R	6	R
1788	>64	R	6	R
1990	>64	R	10	R
2058	>64	R	6	R
2063	>64	R	6	R
2069	>64	R	6	R
2200	>64	R	9	R
2209	>64	R	7	R
2224	4	R	19	R
2334	4	R	25	S

2447	>64	R	7	R
2463	>64	R	11	R
2476	4	R	23	S
2497	0.25	S	30	S
2564	64	R	11	R
2568	>64	R	7	R
2587	>64	R	7	R
2645	>64	R	6	R
2741	32	R	15	R
2786	>64	R	10	R
2864	>64	R	11	R
2895	32	R	17	R
2948	>64	R	13	R
3000	32	R	15	R
3109	>64	R	6	R
3185	>64	R	6	R
3270	>64	R	6	R
3323	8	R	21	S
3389	64	R	12	R
3396	>64	R	6	R
3462	0.25	S	6	R
3501	16	R	6	R
3635	32	R	18	R
3666	64	R	13	R
3715	>64	R	6	R
3718	64	R	6	R
3734	64	R	17	R
3760	>64	R	9	R
3807	64	R	13	R
3826	64	R	17	R
3832	0.06	S	16	R
3854	>64	R	12	R
4129	≤0.06	S	28	S
4171	≤0.06	S	28	S
4179	≤0.06	S	26	S
4184	≤0.06	S	29	S
4197	0.5	S	29	S
4214	≤0.06	S	24	S
4228	0.125	S	29	S
4238	≤0.06	S	29	S
4246	0.125	S	31	S
4248	≤0.06	S	26	S

4249	0.5	S	28	S
4257	≤0.06	S	29	S
4263	0.125	S	29	S
4265	0.125	S	30	S
4275	0.125	S	30	S
4277	0.125	S	29	S
4279	0.125	S	33	S
4292	4	R	20	S
4297	2	R	24	S
4333	4	R	16	R
4378	8	R	22	S
4387	16	R	22	S
4408	>64	R	30	S
4852	>64	R	6	R
4855	>64	R	6	R
4859	>64	R	6	R
4860	>64	R	6	R
4861	64	R	12	R
4862	>64	R	6	R
4864	>64	R	6	R
4865	>64	R	6	R
4869	>64	R	6	R
4871	>64	R	6	R
4887	>64	R	9	R
4939	>64	R	6	R
4958	1	S	25	S
4980	0.25	S	12	R
4982	>64	R	6	R
4998	64	R	6	R
5506	>64	R	6	R
5509	64	R	10	R
5510	0.125	S	26	S
5511	>64	R	6	R
5518	>64	R	6	R

Annex 5

Graphics relating minimum inhibitory concentrations with inhibitory zones for each antibiotic. (CTZ) Ceftazidime; (CTA) Cefotaxime; (MER) Meropenem); (IMI) Imipenem/Meropenem; (ERT) Ertapenem/Meropenem.



Annex 6

All resistant genes and each Kp strain that contains ESLB and CARB enzyme genes.

$\mathit{bla}_{\text{TEM-10}}$	$\mathit{bla}_{\text{GES-1}}$	$\mathit{bla}_{\text{CTX-M-15}}$	$\mathit{bla}_{\text{CTX-M-2}}$	$\mathit{bla}_{\text{CTX-M}}$	$\mathit{bla}_{\text{CTX-M-1}}$	$\mathit{bla}_{\text{CTX-M-8}}$	$\mathit{bla}_{\text{SHV-2}}$	$\mathit{bla}_{\text{BEL-1}}$	$\mathit{bla}_{\text{CTX-M-32}}$	$\mathit{bla}_{\text{SHV-12}}$	$\mathit{bla}_{\text{KPC-3}}$	$\mathit{bla}_{\text{GES-5}}$	$\mathit{bla}_{\text{OXA-181}}$
Kp 684	Kp 748	Kp 888	Kp 1094	Kp 1495	Kp 1528	Kp 4861	Kp 2224	Kp 4297	Kp 4859	Kp 4998	Kp 2564	Kp 4297	Kp 5506
Kp 898		Kp 997	Kp 1528					Kp 4887	Kp 4864		Kp 2895	Kp 4887	Kp 5511
Kp 2334		Kp 1031	Kp 1990								Kp 3109		
Kp 4292		Kp 1144									Kp 3389		
Kp 4297		Kp 1507									Kp 3396		
Kp 4333		Kp 1675									Kp 3462		
Kp 4278		Kp 1677									Kp 3666		
Kp 4387		Kp 1788									Kp 3718		
		Kp 2058									Kp 3734		
		Kp 2069									Kp 3760		
		Kp 2200									Kp 3807		
		Kp 2209									Kp 3826		
		Kp 2447									Kp 3854		
		Kp 2463									Kp 4852		
		Kp 2568									Kp 4887		
		Kp 2587									Kp 4982		
		Kp 2645									Kp 5509		
		Kp 2786									Kp 5518		
		Kp 2864											
		Kp 3185											
		Kp 3270											
		Kp 3396											
		Kp 3715											
		Kp 4855											
		Kp 4860											
		Kp 4862											
		Kp 4865											
		Kp 4869											
		Kp 4871											
		Kp 4939											
		Kp 4980											
		Kp 5506											
		Kp 5511											