2015

DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação da Professora Doutora Gabriela Jorge da Silva (Faculdade de Farmácia, Universidade de Coimbra) e da Professora Doutora Emília Duarte (Departamento de Ciências da Vida, Universidade de Coimbra)

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The experimental work of the present study was conducted at the Microbiology Laboratory at the Faculty of Pharmacy of the University of Coimbra, with scientific orientation of Professor Gabriela Jorge da Silva. Professor Emília Duarte was my tutor at the Department of Life Sciences.

Part of this study was presented as poster (C-094b - First Detection of KPC-2 Carbapenemase in *Klebsiella pneumoniae* ST15 and ST348 in Portugal: a Nosocomial Outbreak) at the 54th International Conference on Antimicrobial Agents and Chemotherapy (ICAAC), held in Washington, DC, USA from 5-9, September, 2014.

This work was supported financially by the project PEst-OE/SAU/UI0177/2014 from the Center for Pharmaceutical Studies, University of Coimbra, Portugal.

ACKNOWLEDGMENTS

The success of any kind of work requires the involvement of all people who surrounding us, thus I would like to thank for all who contributed for my studies.

To lord, because in him everything is possible!

Special thanks to my supervisor Prof. Gabriela Jorge da Silva (Faculty of Pharmacy, University of Coimbra) for accepting me to join her research and for all assistance provided during my research period including review of the whole work.

To Prof. Emília Duarte, my internal tutor and coordinator of the master program, I thank for the valuable assistance provided, starting from the early stage of my application and also for her availability and advices during the whole program.

To my employer, Manhiça Health Research Centre-Centro de Investigação em Saúde de Manhiça (CISM), Maputo, Mozambique, my special thank for funding my master studies. To Inácio Mandomando, Betuel Sigaúque and the scientific board of CISM thank for their technical and scientific mentoring at CISM.

To my wife, Antónia Joyce Cumbe, thank for her understanding and all support provided during my stay abroad. Thank you for the little baby, shall she be blessed!

To my late father, Carlos Jamo Vubil, shall your soul rest in peace! And to my mother Rosalina Uaquiço Banze, thank for the immensurable contribution in all stages of my life.

My thanks are also extended to Rui Figueiredo (PhD student), Nuno Mendonça (PhD) and Sandra Ferreira (Lab Technician), for their support and friendship during my research period at the Microbiology Laboratory, Faculty of Pharmacy-University of Coimbra.

Many thanks for all!

DEDICATION

To my late father, **Carlos Jamo Vubil**, shall your soul rest in peace!

LIST OF ABBREVIATIONS

AmpC- Ampicillinase C

ACT- AmpC Type

BLAST- Basic Local Alignment Search Tool

CC- Clonal Complex

CTX-M- Cefotaximase

CMY- Cephamycin

CLSI- Clinical and Laboratory Standards Institute

DHA- Dhahran Hospital, Saudi Arabia

DNA- Deoxyribonucleic Acid

DDST- Double Disc Synergy Test

ESBL- Extend Spectrum Beta-Lactamase

EDTA- Ethylene Diamine Tetracetic Acid

EUCAST- European Committee for Antimicrobial Susceptibility Testing

FOX- Cefoxitin

GES- Guiana Extend Spectrum β-lactamase

IMP- Imipenemase

IMI- Imipenem hydrolyzing β-lactamase

Inc- Incompatibility

KPC- *Klebsiella pneumoniae* carbapenemase

kb- kilo base

MBL- Metallo Beta-Lactamase

MHT- Modified Hodge Test

MLST- Multi-Locus Sequence Typing

NDM- New Delhi Metallo Beta-lactamase

NMC- Non Metalloenzyme Carbapenemase

OXA- Oxacillinase

PCR- Polymerase Chain Reaction

PBS- Phosphate Buffered Saline

PBP- Penicillin Binding Protein

PBRT- PCR Based Replicon Typing

PFGE- Pulsed Field Gel Electrophoresis

rpm- rotation per minute

SHV- Sulphydryl Variable

ST- Sequence Type

SME-*Serratia marcescens* enzyme

TEM- Timoniera

TBE- Tris Borate EDTA

TE- Tris-EDTA

TSA- Trypticase Soy Agar

TSB- Trypticase Soy Broth

UV- Ultra Violet

VIM- Verona Integron Metallo Beta-lactamase

FIGURES

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Appendix 1. Primers sequences used for detection of β-lactamases.

Appendix 2. Primers used for MLST of *K. pneumoniae* (Diancourt *et al*., 2005).

Appendix 3. Primers used for identification of Plasmid Inc groups (Carattoli *et al*., 2005).

ABSTRACT

The emergence and spread of antibiotic resistant bacteria is a global concern and represents a major threat for patient care. Of particular importance is the dissemination of β-lactamases with ability to inactivate almost all β-lactam antibiotics, leading to the limitation of treatment options. Therefore, rapid detection and characterization of resistant determinants is an important tool for patient management and improvement of infection control measures.

The present study aimed to characterize genetically a collection of isolates of *Klebsiella pneumoniae* ertapenem resistant recovered from patients at the University Hospital of Coimbra from January-December, 2013. All isolates were tested for antimicrobial susceptibility by disk diffusion method. Conventional PCR was used to detect β-lactamases genes and for plasmid typing while clonal relatedness of the isolates was carried out by PFGE and MLST analysis. Finally, plasmid conjugation assays were conducted to verify the potential of transfer of antibiotic resistance determinants among bacteria.

A total number of 27 non-duplicated *K. pneumoniae* isolates have been analyzed and the majority was recovered from urine 48.1% (13/27) and blood 25.9% (7/27). A high prevalence of antibiotic resistance associated with production of β-lactamases, including to carbapenems, was observed. Extended spectrum β-lactamases (ESBLs) was the more frequent resistance mechanism found, with TEM-type β-lactamase (77.7%) being the most common found followed by CTX-M-type (70.3%). KPC- carbapenemase was detected in 66.6% of the isolates. This is the first report of KPC-2 in nosocomial isolates of *K. pneumoniae* in the referral University Hospital of Coimbra and in Portugal. KPC was associated with conjugative IncF plasmid type which supports their dissemination and potential spreading to other bacterial.

PFGE analysis showed a limited number of clones represented by three MLST profiles, ST11, ST15 and ST348, which suggest the presence of specific clones in the hospital. Continuous surveillance of antibiotic resistance and rational use of antibiotics is needed to improve patient management and infection control measures.

Key words: *K. pneumoniae*, antibiotic resistance, β-lactamases.

RESUMO

A emergência e disseminação de bactérias resistentes aos antibióticos é um problema global e representa uma grande ameaça para a saúde dos pacientes. Com importância especial é a disseminação de β-lactamases com capacidade de inativar quase todos os antibióticos β-lactámicos contribuindo para limitação das opções de tratamento. Portanto uma rápida deteção e caracterização dos determinantes de resistência constituem uma ferramenta importante para o tratamento dos pacientes e melhoramento das medidas de controlo de infeções.

O estudo teve como objetivo caracterizar geneticamente isolados de *Klebsiella pneumoniae* resistentes ao ertapenem de pacientes admitidos nos Hospitais da Universidade de Coimbra, de Janeiro a Dezembro de 2013. Os isolados foram testados a sua suscetibilidade aos antibióticos usando o método de disco difusão. A PCR convencional foi usada para deteção de genes que codificam as β-lactamases e para a tipagem dos plasmídeos, enquanto a relação clonal dos isolados foi determinada com recurso as técnicas de PFGE e MLST. Os ensaios de conjugação foram feitos para avaliar o potencial de transferência dos genes de resistência entre bactérias.

Foram analisados 27 isolados de *K. pneumoniae* não duplicados e a maioria foi de urina 48.1% (13/27) e sangue 25.9% (7/27). Foi observada uma alta taxa de resistência aos antibióticos associada a produção de β-lactamases. As beta-lactamases de espectro estendido (ESBL) foram o principal mecanismo de resistência encontrado, com TEM (77.7%), o mais prevalente seguido de CTX-M (70.3%). A carbapenemase-KPC foi detetada em 66.6% dos isolados. Este é o primeiro reporte de KPC-2 em isolados nosocomiais de *K. pneumoniae* nos hospitais da Universidade de Coimbra, e em Portugal. A KPC estava associada a um plasmídeo conjugativo do grupo F oque sustenta a sua disseminação e capacidade de transmissão para outras bactérias.

Análises de PFGE mostraram um número limitado de clones representados por três diferentes perfis de MLST, ST11, ST15 e ST348, oque sugere a presença de clones específicos no hospital. Uma contínua vigilância de resistências aos antibióticos e uso racional dos antibióticos é necessária para melhorar o tratamento dos pacientes e para o controlo das infeções.

Palavras-chave: *K. pneumoniae*, resistência aos antibióticos, β-lactamases.

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1. INTRODUCTION

Klebsiella pneumoniae is an important Gram negative bacterial pathogen responsible for several infections ranging from soft tissue lesions to life threatening conditions like pneumonia. These bacteria can cause both community and hospital acquired infections [1], being one of the most important nosocomial pathogens [2].

Currently, there is a worldwide problem of emerging multi drug resistant *K. pneumoniae* isolates which leads to the limitation of treatment options and makes infection control difficult. Of particular importance is the increasing of resistance to β-lactams since these antibiotics are used as a choice to treat most of bacterial infections. Therapeutic challenges associated with these highly resistant strains correlate with higher morbidity and mortality, increased length of hospitalization, and an overall increase in health care costs [3].

Resistance to β-lactams can be afforded either by changes on the penicillin binding proteins (PBP), diminished permeability of outer membrane (OM), active efflux or by drug inactivation by β-lactamases [4]. However, the production of β-lactamases represents the most important mechanism of resistance to β-lactam antibiotics among Gram-negative bacteria. The β-lactamases encoding genes are usually carried on mobile genetic elements which facilitate their dissemination among bacterial species.

Up to date, more than 900 different β-lactamases have been recognized [5]. The most clinically important, and widely disseminated, are the extended-spectrum β-lactamases (ESBLs) of the CTX-M, TEM and SHV families, however, other β-lactamases families have also emerged, such as plasmid-mediated AmpC and carbapenemases [6].

The production of ESBLs is the most common mechanism of resistance to third-generation cephalosporins in *Enterobacteriaceae*. ESBLs are mostly plasmid mediated β-lactamases that efficiently hydrolyzes oxyimino-cephalosporins and monobactams but they are inhibited by β-lactamases inhibitors such as clavulanic acid [7].

ESBLs are increasingly reported worldwide and have been linked to successful Enterobacteria clones possessing great epidemic potential [7]. On the other hand, plasmids carrying ESBL determinants may carry additional β-lactamases genes as well as genes conferring resistance to other antimicrobial classes [8].

A variety of genetic mobile elements such as transposons and insertion sequences play an important role in the dissemination of ESBL genes. TEM-type ESBLs are acquired by mutation of plasmid mediated parent TEM-1 and TEM-2 genes, being *Escherichia coli* the main producer of TEM-type ESBL while SHV-types ESBLs are the derivatives of chromosomal parent SHV-1 gene which occur mainly in *K. pneumoniae* and are acquired by insertion sequences from chromosome to plasmid [9]. In contrast to TEM and SHV ESBLs, CTX-M genes originated from the chromosomal β-lactamase genes of *Kluyvera* spp., which are environmental bacteria found worldwide, and are captured mainly by insertion sequence elements [10].

The CTX-M-type β-lactamases derive their name from the efficient cefotaximase activity against cefotaxime, which is a functional hallmark of these enzymes. The most common hosts of CTX-M-type enzymes are the species of *E. coli* and *K. pneumoniae*, however, they have been also detected in many other Enterobacteria species including *Salmonella enterica*, *Shigella* spp., *Klebsiella oxytoca*, *Enterobacter* spp., *Pantoea agglomerans*, *Citrobacter* spp., *Serratia marcescens*, *Proteus mirabilis*, *Morganella morganii* and *Providencia* spp. [11].

Several variants of the CTX-M enzymes have been described, being CTX-M-15 and CTX-M-14 the most common sequence types detected worldwide in clinical important pathogens followed by CTX-M-2, CTX-M-3 and CTX-M-1. The high prevalence of CTX-M might be associated with a conjugative plasmid-mediated horizontal transfer and clonal spreading [12].

Like ESBLs, AmpC mediated β-lactamases have a broad substrate profile that include penicillins, cephalosporins (including cephamycins) and monobactams, however, they are resistant to β-lactamases inhibitors [13]. AmpC enzymes can be either chromosomal or plasmid encoded. Plasmid mediated AmpC genes are found in nosocomial isolates of *E. coli* and *K. pneumoniae* and they are derived from the chromosomal AmpC genes of several members of the *Enterobacteriaceae* family like *Enterobacter cloacae*, *Citrobacter freundii*, *M. morganii* and

Hafnia alvei [14]. These enzymes comprises mainly the CMY, ACT, FOX and DHA types being CMY type the most prevalent and distributed worldwide [15].

Carbapenems are antibiotics of choice for treatment of infections due to Gram negative multi drug resistant ESBL producing organisms. However, carbapenem resistant strains have been reported [16, 17] and have emerged as a major cause of nosocomial infections worldwide. Therefore, detection and surveillance of carbapenem resistant organisms have become a matter of major importance for the selection of appropriate therapeutic schemes and the implementation of infection control measures.

The most important carbapenemases are categorized as three types of enzymes: (I) the KPC type, (II) the VIM, IMP, and NDM metallo-β-lactamases and (III) the OXA-48 type enzymes [18]. However, the production of *Klebsiella pneumoniae* carbapenemase (KPC) is being reported as the most common mechanism for carbapenem resistance in *Enterobacteriaceae* isolates [19].

KPC is a plasmid encoded beta-lactamase firstly reported from *K. pneumoniae* in the United States and then worldwide [20]. Currently, KPC has been also found in several other species including *E. coli*, *Enterobacter* spp., *Salmonella enterica*, *Proteus mirabilis*, *C. freundii*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [21].

The rapid worldwide increasing spread of *Enterobacteriaceae* harboring KPC-carbapenemase represents a great concern and might correlate with the location of KPC encoding gene on plasmid with the potential for spreading among other bacterial species. KPC is carried in a Tn3-type transposon, Tn4401, a genetic element which is capable of inserting into diverse plasmids of Gram-negative bacteria. Plasmids carrying KPC are often also associated with resistance determinants for other antibiotics [22].

Different variants of KPC based on their nucleotide sequence have been described, with KPC-2 and KPC-3 being the most frequently found [23]. Studies on molecular epidemiology of KPC producing bacteria have revealed that few lineages have been responsible for dissemination of KPC encoding gene being sequence type 258 (ST258), the most dominant genotype [20].

Metallo-β-lactamase-mediated resistance in Gram negative bacteria is emerging worldwide and is mostly due to the mobilization of IMP and VIM-type MBL [24] being more frequently associated with *P. aeruginosa* than *Enterobacteriaceae*. Most of transferable MBL are commonly encoded by genes carried in integrons, which are segment of mobile DNA that can capture genes by site-specific recombination, being class I integrons the most reported and well characterized [25].

The NDM-1 β-lactamase is a novel type of MBL described in 2009 in India from a patient who acquired urinary tract infection due to carbapenem resistant *K. pneumoniae*, which was resistant to all antibiotics tested except colistin, and since then, it has been reported in an increasing number of infections in patients from India, Pakistan and the United Kingdom [26]. The NDM-1 encoding gene was found located on different large plasmids (a 180-kb plasmid for *K. pneumoniae* and a 140-kb for *E. coli*) which are easily transferable to susceptible *E. coli* J53 strains with high frequency [27]. To date NDM-1 carbapenemase has been found in different *Enterobacteriaceae* species, namely *C. freundii*, *E. cloacae* and *M. morganii* [26].

The plasmid born OXA-48 carbapenenemase was firstly described in epidemic *K. pneumoniae* isolates from Turkey, and then reported in many other countries [28]. Apart of *K. pneumoniae*, this enzyme was also detected in other species like *E. coli*, *E. cloacae*, *C. freundii* and *Providencia rettgeri* [29].

In Portugal, like in several other countries, there is a dramatically increasing in antibiotic resistance, especially for β-lactams, putting a serious threat for patients care. Several studies have analyzed the dissemination of ESBLs [30-33] and AmpC mediated beta-lactamases among *Enterobacteriaceae* [32, 34], however, there are limited data concerning carbapenemases producing organisms. One study showed an occurrence of KPC-3 in isolates of *K. pneumoniae* recovered from Hospital wards in Lisbon [35]. Ertapenem resistant *K. pneumoniae* strains were previously reported at the University Hospital of Coimbra, Portugal, but the resistance to this carbapenem was mainly associated with deficiencies in major porins [36]. However, the preferential use of carbapenems to treat multi-drug resistant ESBL bacterial infections amplifies

the risk of further expansion of carbapenemases producing organisms, highlighting the importance of continuous surveillance of antibiotic resistance.

Therefore, the present study proposed to identify and characterize the dissemination of carbapenemases and other β-lactamases among isolates of *K. pneumoniae* resistant to ertapenem recovered from patients admitted to the University Hospital of Coimbra, Portugal.

Gaining a good understanding of molecular basis for development of antibiotic resistance is an important key for selection of suitable therapy, to improve patient management and for infection control measures and also to create new strategies for development of new treatment options.

1.1. Antibiotic Resistance: A Global Concern

The discovery of antibiotics have revolutionized the history of medicine in several aspects, and since then, contributed to reduce the number of deaths due to infectious diseases worldwide, and representing a shift point in the history of humankind.

Since the first introduction of sulfa drugs and penicillin into clinical use in 1930s and 1940s, respectively, large numbers of antibiotics have been developed and hence contributed to human health. The called era of antibiotics led to optimism that infectious diseases can be controlled and prevented, and confidence that modern medicine would prevail against infectious diseases [37]. However, in contrast to this optimism, infectious diseases are still the second leading cause of death worldwide [38] causing over 13 million deaths each year [37].

Worldwide, antibacterial resistance has increased dramatically and is currently recognized as a major medical challenge. Mortality attributed to resistance is considerable and thus contributes to the infectious diseases burden. In the European Union, each year, about 25.000 patients die from an infection with the selected multidrug resistant bacteria, while more than 63.000 patients in the United States die every year from hospital acquired bacterial infections [39].

Antimicrobial resistance was recognized soon after the deployment of sulfonamides and penicillin [40] and now it appears that the emergence of antibiotic resistance bacteria is inevitable to most very new drugs. The global public problem has been caused by emerging infections not yet recognized, re-emerging infections experienced previously that have reappeared in more virulent forms and from antimicrobial resistant bacterial infections [38].

Several factors play a role for the global burden of antimicrobial resistance, however, the consumption of antibiotics combined with the level of compliance with infection control measures has been suggested as the drive force for antibiotic resistance globally [41, 42]. It has been estimated that the human gut contains 10^{13}-10^{14} bacteria, 10 fold more than the total human cells in the body [43] and thus, bacteria that belong to the normal flora in humans become indiscriminately exposed to selection pressure every time antibiotics are used. Therefore, the most significant resistance has been emerging from opportunist pathogens [41].

Once the resistance has been selected, rapid human-to-human transmission of pathogens with resistance genes enables their spread. Although awareness is increasing, basic infection control measures such as hand hygiene are still performed suboptimal in many settings. On the other hand, patient isolation and cohort measures are often delayed or omitted by delays in diagnosis or due their relative costs [42].

Among other factors, the globalization has shifted the balance toward the emergence and uncontrolled spread of resistance. International travel, adoption, trade and immigration all facilitate the globalization of antimicrobial resistance [44].

In addition to the steadily increasing consumption of antibiotics to treat illnesses, the uses of antibiotics as growth promoters in livestock production [42, 45], in agriculture, aquaculture and in industry, as well as the uncontrolled release of antibacterial compounds into the environment have immensely increased the selection pressure [5]. However, it is known that naturally occurring antibiotics are produced by microorganisms and thus, it is true that microorganisms might develop a counteract system for their survival. This supports the hypothesis that antibiotic resistance is also an ancient and naturally occurring phenomenon. Soil is generally one of the largest and most diverse microbial habitats on earth and is recognized as a vast repository of antibiotic resistance genes [46].

Thus, combined strategies are needed to prevent the emergence and dissemination of antibiotic resistant strains. The World Health Organization (WHO) recommends a continuing surveillance systems, rational use of antibiotics and infection control measures as well as efforts to develop new antibacterial agents are needed [47].

1.2. Bacterial Mechanisms of Antibiotic Resistance

Antibiotic resistance is defined as the ability of bacteria to not be inhibited by the usually achievable systemic concentration of an agent that is administered according to the normal dosage schedule [48].

Bacterial antibiotic resistance can be attained through intrinsic or acquired mechanisms. Intrinsic mechanism is a characteristic of particular bacterial species and is specified by natural occurring genes found in the host chromosome [49]. This passive resistance is a consequence of general adaptive processes that are not necessarily linked to the use or a given class of antibiotics [50].

Acquired resistance is the active major mechanism of antimicrobial resistance and is the result of specific evolutionary pressure to develop a counterattack mechanism against an antimicrobial agent or a class of antimicrobials, so that bacterial populations previously susceptible to an antimicrobial become resistant [51]. Acquired resistance can be passed vertically to daughter cells or by horizontal transfer of resistance genes among strains and species through the processes of transformation, conjugation and transduction [50].

The major mechanisms of active antimicrobial resistance are: (I) prevention of accumulation of antimicrobials either by decreasing uptake or increasing efflux of antimicrobial from the cell through a collection of membrane-associated pumping proteins; (II) qualitative drug target site alteration by mutation, which reduces the affinity for antimicrobial; and (III) inactivation of antibiotics either by hydrolysis or by modification [52].

1.2.1. Alteration of Outer Membrane Permeability and Efflux Pumps

The outer membrane of Gram-negative is a barrier for hydrophilic compounds. The entry of cytoplasmic targeted compounds is usually achieved through carrier-mediated transport mechanisms or by channels in the outer membrane formed by porins. Antibacterial compounds transported in this way may be subject to resistance by loss of non-essential transporters, by lack

of porins or by mutations that are able to modify the structure of these channels and thus decreasing the influx [52].

Efflux pumps are transmembrane transporter proteins used by bacterial cell to pump out toxic compounds including diverse antibiotics [50]. Efflux pumps can be specific to an antibiotic however, most of them are multi-drug transporters that are capable to pump a wide range of unrelated antibiotics, and thus, significantly contribute to multi drug resistance [53].

Antibiotic active efflux is relevant for antibiotics that act inside the bacterial cell and takes place when the microorganism is capable of developing an active transport mechanism that pumps out the antibiotic molecules that penetrate into the cell [40]. Increasing the efflux plays a role, especially with hydrophobic compounds that presumably enter the cell through diffusion [50]. At the same speed where the antimicrobials are entering the cell, efflux mechanisms are pumping them out, before them reach their target [50]. This means that efflux transport mechanism must be more efficient than the influx mechanism in order to be effective [40].

1.2.2. Alteration of Drug Target

The interaction between an antibiotic and the target molecule is very specific, so any alteration of the target can influence the antibiotic binding affinities. Target modification is a common mechanism of resistance resulting from natural variations or acquired changes in the target sites of antimicrobials that prevent drug binding or action. Natural variations result from spontaneous mutation of a bacterial gene on the chromosome and selection in the presence of the antimicrobial [54]. But the most common mechanism of target alteration is the acquisition of new genes carried on plasmids or transposons that result in enzymatic modification of the normal target so that it does not bind to the antibiotic [52].

1.2.3. Enzymatic Inactivation or Modification

Enzymatic inactivation either by hydrolysis or by modification is the major mechanism of resistance in pathogenic bacteria to natural antibiotics such as beta-lactams, aminoglycosides and chloramphenicol [52]. There are three main enzymes that inactivate antibiotics: beta-lactamases, aminoglycoside-modifying enzymes and chloramphenicol acetyltransferases [53].

Beta-lactamases are enzymes with ability to hydrolyze the beta-lactam antibiotic ring and they represent the most common mechanism of resistance for this class of antibacterial agents in clinically important Gram-negative bacteria. Resistance to beta-lactams arises from natural mutation of chromosomal genes or from acquisition of extrachromosomal genetic elements like plasmids or transposons while resistance to aminoglycosides is primarily based on a chemical modification of the aminoglycoside which compromises binding of the target to it ribosomal subunit [55].

The group of transferases are enzymes which covalently modify antibiotic leading to structural alterations that impair target binding [50]. Resistance to chloramphenicol is mainly afforded by acetyltransferases [52].

1.3. Mechanisms of Bacterial Horizontal Gene Transfer

Treatment of bacterial infections is been compromised by the emergence of bacterial pathogens resistant to multiple antibiotics. Acquisition of multi-drug resistance is mostly associated with horizontal transfer of resistance genes among bacterial species through mobile genetic elements harboring resistance determinants.

Mobile genetic elements (MGE) are defined as DNA segments which move within genomes (intracellular mobility) or between bacterial cells, also known as intercellular mobility[56]. Intercellular exchange of DNA can be afforded by the processes of transformation, conjugation and transduction [50].

Natural transformation of bacteria was the first mechanism of prokaryotic horizontal gene transfer to be discovered. It consists on the active uptake by a cell of free (extracellular) DNA (plasmid and/or chromosomal) and the heritable incorporation of its genetic information between closely related bacteria [57].

Bacterial conjugation is one of the main mechanisms for horizontal gene transfer, it constitutes a key element in the dissemination of antibiotic resistance and virulence genes to human pathogenic bacteria[58]. It requires independently replicating genetic elements, usually, conjugative plasmids. These genetic elements encode proteins that facilitate their own transfer from the donor plasmid-carrying cell to a recipient cell [56] and a physical stable contact must be established between the donor cell and the recipient to allow the transfer of DNA [57].

Finally, gene transfer by transduction is mediated by independently replicating bacterial virus called bacteriophages or phages. The transduction process involves the infection of the bacterial cell, the mobilization and incorporation of bacterial genes into the phage genome and their transmission to another host bacterial cell [40].

1.4. Classification of β-Lactamases

Two classification schemes of beta-lactamases are currently in use namely, functional and molecular classification, being the molecular classification proposed by Ambler (1980) the most widely used [59].

The molecular classification is based on the amino acid sequence and divides beta-lactamases into four classes (A-D). Classes A, C and D enzymes utilize serine in their active site for beta-lactam hydrolysis while class B metalloenzymes (Metalo beta-lactamases-MBL) require divalent zinc ions for substrate hydrolysis [60]. Class A is usually referred as penicillinases, but it also includes other enzymes like ESBL and some carbapenemases, while class C comprises cephalosporinases (AmpC) and class D are known as oxacillinases.

Functional classification by Bush (1995) takes into account substrate and inhibitor profiles in an attempt to group the enzymes in ways that can be correlated with their resistance phenotype in clinical isolates [60].

1.5. Detection of ESBL and AmpC β-Lactamases

The double disk synergy test (DDST) is recommended as the confirmatory phenotypic test for detection of ESBLs among *Enterobacteriaceae* in which clavulanic acid containing disk is placed in the center between two third generation cephalosporins [48]. Nevertheless, chromogenic media, such as the chromID ESBL (bioMérieux) and Brilliance ESBL agar (Oxoid) have become commercially available [61].

Challenging the detection of ESBLs producing organisms is the coproduction of an AmpC beta-lactamase. This enzyme is resistant to clavulanic acid and the presence of an AmpC beta-lactamase in Gram negative bacteria might potentially cause false negative reporting of ESBLs producers, therefore, a number of detection methods for AmpC have been proposed. Derepressed chromosomal or plasmid-mediated AmpC producers can be distinguished from ESBLs by the fact that these confer resistance to cephamycins, like cefoxitin and cefotetan.

Reduced susceptibility to cefoxitin in the *Enterobacteriaceae* is recommended as an indicator of AmpC activity, however cefoxitin resistance may also be mediated by alterations to outer membrane permeability [62]. In the other hand, enzyme extraction methods have been suggested as the optimum phenotypic detection method for AmpC activity but they are labor intensive and are not suitable for routine clinical use [63]. Inhibitors of the AmpC enzyme are also described and include boronic acid [64] and cloxacillin [65]. The use of disk approximation tests by Kirby-Bauer testing to detect inducible AmpC activity have been also described using one antibiotic as an inducing substrate and a second antibiotic as a reporter substrate [66]. However, PCR based methodologies remain the gold standard for detection of both ESBLs and AmpC producing organisms.

1.6. Detection of Carbapenemases

The detection of carbapenemase producers in clinical infections is based first on susceptibility testing results obtained by disk diffusion or by automated systems [67]. However, low level resistance and even susceptibility to carbapenems have been observed for producers of any type of carbapenemases [68]. Thus, specific tests for carbapenemases detection from phenotypic screening to advanced molecular techniques have been developed.

The clover leaf method or modified Hodge test (MHT) has been extensively used as a general phenotypic method for the detection of carbapenemase activity. It is based on the inactivation of a carbapenem by carbapenemase-producing strains that enable a carbapenem-susceptible indicator strain to extend growth toward a carbapenem-containing disk, along the streak of inoculum of the tested strain [69]. The assay is, overall, sensitive for the detection of a carbapenemase-mediated mechanism of resistance to carbapenems [67] and multiple isolates can be tested in a single Mueller-Hinton agar plate [70]. However, the test is time consuming and may lack of specificity (false positive strains when ESBL and AmpC are associated to porin loss) and lack of sensitivity for detection of VIM and NDM producers [71] and does not provide information regarding the type of carbapenemase involved. Moreover, there might be some difficulties in interpretation of the cloverleaf test for weak carbapenemases producers, particularly for MBLs *Enterobacteriaceae* [61, 67]. MHT was found to be useful for detection of KPC and OXA-like producers [68].

Boronic acid–based inhibition testing is reported to be specific for KPC detection in *K. pneumoniae* when performed with imipenem or meropenem but not with ertapenem if corresponding isolates co-produce a plasmid mediated AmpC β-lactamase [68].

Several inhibitor-based tests have been developed for specific detection of MBL producers. They are based on the synergy between MBL inhibitors such as EDTA and dipicolinic acid and a carbapenem (imipenem and/or meropenem) and/or an oxyimino-cephalosporin (ceftazidime) as indicator beta-lactam compounds [67]. These tests take advantage of the metalloenzyme dependence on zinc ions, and use the chelating agents to inhibit β-lactam hydrolysis. Several

formats like the disk diffusion or broth dilution of EDTA based synergy tests have been the most commonly used and evaluated [67].

Spectrophotometric measurement of carbapenem hydrolysis is considered to be the reference standard method for detection of carbapenemase production in a suspected organism [67]. However, this assay is time consuming, requires specific training and equipment, and does not easily discriminate between different types of carbapenemases [68].

Molecular biology techniques usually PCR and sequencing based methodologies are the gold standard for the identification of carbapenemases. The disadvantages of these molecular-based technologies include their cost, the requirement of trained personnel and the absence of detection of any novel genes.

1.7. Methods for Molecular Typing of Bacterial Pathogens

Understanding bacterial diseases and the development of measures to counter its spread, depends on the ability to characterize bacterial pathogens [72]. Typing is referred as the identification of different types of organisms within a species [73]. The main role of microbial typing is to assess the relationship among microbial isolates which is important to recognize outbreaks of an infection, to detect cross transmission of nosocomial pathogens, to determine the source of infection and to recognize particularly virulent strains among organisms [74]. Bacterial typing has also greatly contributed to increase the effectiveness of surveillance systems and has provided meaningful advances to public health control measures.

A variety of bacterial typing systems are currently available that vary greatly with respect to effort required, cost, reliability and ability to discriminate between bacterial strains. Traditional bacterial typing systems based on phenotype such as serotyping, biotype, phage-type, or antibiogram have been used for many years to study the epidemiology of bacterial infectious diseases [75, 76]. However, phenotypic based methodologies are generally variable, labor intensive and time consuming to be of practical value in epidemiological investigations [77].

To overcome these limitations, molecular based techniques, which rely on distinguishing genetic variations among isolates, have been established. These methods can be categorized in three groups, (1) DNA banding pattern, (2) DNA sequencing and (3) DNA PCR/hybridization. DNA banding pattern based-genotyping methods discriminate the isolates based on differences in the size of DNA fragments generated by the amplification of genomic DNA or by cleavage of DNA through restriction enzymes [78]. DNA sequence-based genotyping generate the original sequence of nucleotides and discriminate among bacterial strains directly from polymorphisms in their DNA[78, 79], while DNA PCR/hybridization methods relies on the amplification of genetic particular target [79] and or hybridization with specific probes [78]. However, the use of emerging technologies such as the Matrix Assisted Light Desorption Ionization Time Of Flight (MALDI-TOF) mass spectrometry are being developed to facilitate bacterial genotyping in routine analysis [80] in which the mass spectra of bacterial whole cells can be measured, compared and put into framework of molecular diversity.

Herein, the principles and applications of Pulsed Field Gel Electrophoresis (PFGE), Multi-Locus Sequence Type (MLST) and Plasmid typing systems are discussed in detail. PFGE and MLST are worldwide used typing methods in almost all clinically important bacterial species, to study clonal relatedness, while plasmid typing is a useful tool for identification and characterization of plasmids carrying antibiotic resistance genes.

1.7.1. Pulsed Field Gel Electrophoresis (PFGE)

PFGE is a DNA banding pattern based typing method in which the bacterial genome is digested with rare cutting restriction enzymes which recognize specific DNA sequence of 6-8 bases and generate a smaller number of DNA fragments of a wide range of sizes that can be separated using a specialized electrophoresis apparatus [79], being the contour-clamped homogeneous electric field electrophoresis (CHEF, BioRad), the most used system [76]. The differences in the restriction profiles are used to carry out genetic relatedness among isolates.

The usefulness of PFGE resides in the ability to separate chromosomal DNA fragments of Mega bases in size which is not possible by the conventional agarose gel electrophoresis which is unable to resolve DNA molecules larger than 40-50Kb [76]. Since PFGE provides a high resolution macro-restriction analysis at the genome level, it has been considered as the gold standard method for typing many bacterial species [74, 78].

In general, the principle of PFGE involves immobilizing bacteria, by mixing bacterial cell suspension of a known optical density with melted agarose prior to cell lyses to protect the chromosomal DNA from mechanical damage that can occur with the manipulation of free DNA. The embedded cells are lysed with a detergents (sarcosine) and enzymes (proteinase K) and the released DNA is immobilized in agarose plugs. The agarose plugs are then washed with water and Tris-EDTA (TE) buffer to remove cellular debris and the purified DNA is digested with rare cutting restriction enzyme, being *Xba*I the most common used restriction enzyme for Gram negative bacteria DNA [76]. The plugs containing the immobilized DNA are then added to an agarose gel and subjected to electrophoresis. During electrophoresis, the polarity of the current is switched at a regular intervals allowing separation of larger molecules ranging from 20-800 Kb [79]. The DNA fragments are then visualized by staining the gels with a fluorescent dye such as ethidium bromide and the data analysis can be accomplished through commercially available number of software such as the BioNumerics (Applied Maths).

Since computer assisted analysis may not be accessible for all laboratories, a standardized procedure for interpretation of the PFGE patterns has been proposed by Tenover *et al*., 1995 [81]. Based on the Tenover *et al*. algorithm, bacterial isolates yielding the same PFGE profile are considered as belonging to the same strain. Isolates that differ in a single genetic event, which is reflected by difference of one to three bands, are considered as being genetic closely related, while isolates differing from four to six bands are likely representing two independent genetic events and thus referred as being genetic possibly related. Finally, bacterial isolates containing six or more band differences, which are representative of three or more genetic events, are considered as being unrelated strains. However, this method is only applicable to analyze small

and local studies in which genetic variability is presumed to be limited, typically not greater than 30 isolates [81].

The success of PFGE results from its excellent discriminatory power and high epidemiological concordance, it is also relatively inexpensive [73]. Despite the fact that every genetic change and macro-restriction are not detected, the sum of visible band fragments for the average bacterial PFGE pattern, represents greater than 90% of the total genome, thus PFGE provides a highly visual sense of global chromosome monitoring [76].

Although the discriminatory power which leads to their widely use, PFGE have also several drawbacks. This method is laborious and time consuming, absence of reproducibility and portability due to the subjectivity of interpretation. In addition, some bacterial isolates are not typable by this method and may also lack resolution power to distinguish bands of nearly identical size [78, 79]. However, efforts have been made to improve PFGE data interpretation such as the PulseNet program which establishes a standard operating procedure of PFGE for typing of food borne bacterial pathogens [82].

1.7.2. Multi-Locus Sequence Type (MLST)

Microbial genomes within a species are subject to sequence variability due to mutation or recombination, and the sequence variability in particular genes can be used in molecular typing schemes to determine the relatedness of bacterial population. One of these methods is MLST in which the sequences of multiple conserved genes are compared for nucleotide base changes [79].

MLST is a DNA sequencing based methodology which relies in sequencing of a set of bacterial housekeeping genes, usually seven. Typically, fragments of 450-500 bp are sequenced and each gene sequence variation is assigned an allelic number enumerated according to their discovery, and the combination of the seven alleles is used to determine the sequence type number (ST) through the online MLST (www.mlst.net) available databases [83]. Isolates with identical ST are defined as being clonal by MLST. Additionally, strain relatedness can be carried on by the use of electronically available clustering software such the eBURST (based upon related sequence type)

algorithm which is able to divide the isolates based on their ST into clonal complexes (CC). Using the eBURST clustering, isolates with high level of genetic similarity (such as sharing six to seven identical alleles) are combined as members of the same CC [84], thus isolates in distinct CCs are less genetic closely related to one another than those within the CC [79].

Since the housekeeping genes are functional metabolic genes, they are not subject to strong selective pressures that can lead to relatively rapid sequence changes overtime, thus representing a powerful discriminatory tool to study genetic relatedness among bacterial populations.

Unlike PFGE, the data produced by MLST is unambiguous, highly reproducible and exchangeable among different laboratories through the use of electronically available databases (www.mlst.net). By these reasons, MLST is considered as the reference genotyping methodology for long term global epidemiology and population genetic studies [78].

Although the advantages of MLST, it has also some drawbacks. MLST is based on sequencing of highly conserved housekeeping genes, thus it is possible to lack discrimination of closely related isolates [78] and the sequencing of seven genes, or more, is costly and time consuming [73]. Alleles are assigned to a numbering system that is not representative to the entire genome, therefore, the use of advanced molecular biology methodologies such as the whole genome sequence (WGS) in which the entire bacterial genome is sequenced has been suggested as the future of studying microbial diversity.

1.7.3. Plasmid Typing

Plasmids are defined as circular or linear extrachromosomal double stranded DNA that replicate autonomously in a host bacterial cell, they do not code for essential cellular functions but they can carry genes for virulence or antibiotic resistance traits.

Plasmids are found in virtually all bacterial species and they can spread vertically from the parent to progeny or horizontally from one cell to another [85]. They must replicate, control their copy number, and ensure their inheritance at each cell division [56].

Identification and classification of plasmids is especially important in medicine because genes with clinically important traits, such as drug resistance and virulence factors, are frequently carried on plasmid, and thus, the recognition of the type of virulence or resistance plasmid present in a certain pathogen may be crucial in tracing the source and infection spreading, and it may also serve in establishing laboratory diagnosis [86]. Plasmid typing is also useful for tracing the genetic relatedness and evolutionary origins among bacterial isolates [87].

Naturally occurring plasmids range in size from one to some hundred kilo bases and in copy number from one to several hundred in cell. However, copy number is a fixed characteristic of any plasmid under constant conditions and is controlled by a plasmid coded system that determines the rate of initiation of replication [88]. By contrast, it is impossible for plasmids with the same replication mechanism to coexist in the same bacterial cell, a phenomenon called incompatibility (Inc) [56].

A formal scheme of plasmid classification is based on incompatibility and was developed by Datta & Hedges in 1971 [86]. This procedure relies on the introduction by conjugation or transformation of a plasmid of an unknown Inc group into a cell carrying a known plasmid Inc group. If the resident plasmid is eliminated in the progeny, the incoming plasmid is assigned to the same incompatibility group [89]. Plasmids with the same replication control system are incompatible while plasmids with different replication controls are compatible, so on this basis, plasmids belonging to the same Inc group cannot be propagated in the same cell line [86]. At least 27 Inc groups are recognized among *Enterobacteriaceae* [90].

In 1988, Couturier and colleagues proposed a new plasmid typing scheme based on southern blot hybridization using cloned replication regions as probes to recognize the major plasmids groups among *Enterobacteriaceae* [86]. This approach successfully provided classification of both conjugative and non-conjugative plasmids but the low specificity underestimated the plasmid diversity due to cross hybridization reaction among highly related replicons [91].

In the other hand, the conjugation and hybridization plasmid typing based methodologies are laborious, time consuming and cannot be easily applied to a large scale applications. Thus, a

conventional PCR based replicon typing (PBRT) method was developed by Carattoli *et al*., 2005 to overcome these limitations [89]. This method utilizes a set of five multiplex and three simplex PCR and 18 pairs of oligonucleotides primers to amplify the major plasmid incompatibility groups among *Enterobacteriaceae*, namely: FIA, FIB, FIC, HI1, HI2, I1-Iɣ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA [89].

PBRT is an easy and cheaper plasmid typing tool widely used, though it has several limitations because it does not cover all Inc groups and also cannot detect novel replicons. Other alternative methods, include the use of real-time PCR [85] and DNA sequencing based methodologies have been also described [92].

Another important tool for plasmid typing relies on the ability to perform plasmid extraction from bacterial cells. Like the identification of plasmid Inc groups, plasmid isolation is useful to determine whether a given bacterium carries or not a plasmid [93].

Plasmid extraction can be afforded by the use of commercial available kits such as those from QIAGEN (www.qiagen.com) or by the use of different home based methodologies such as the alkaline lyses with denaturant agents like sodium dodecyl sulphate (SDS) [93-95]. However, limitations include the low yield and the difficulty to extract some bacterial plasmid DNA by the current available methodologies.

1.8. Objectives & Hypothesis

The motivation of the present study was the recent increasing isolation of *K. pneumoniae* and other Enterobacteria with carbapenem resistance at the University Hospital of Coimbra. Although previous studies, which analysed an outbreak of *K. pneumoniae* ertapenem resistant, occurred in 2010 at the same hospital, found that resistance to this carbapenem was associated with alteration in the outer membrane major porins and, not to carbapenemases [36], the recent reports of KPC mediated carbapenem resistance in Lisbon [35] and in the Hospital of Aveiro (Gabriela J. da Silva, personal communication) alerted for an increased risk for the dissemination of this type of carbapenemase into the University Hospital of Coimbra since it is a referral care facility in the country.

Therefore, efforts for surveillance of carbapenamase-producing organisms were continued in 2013 to assess whether the clones of the isolates found in the previous studies were still the same or have already acquired a KPC gene. Thus, the main objective of the present study was to investigate the presence and dissemination of KPC-carbapenemase and other β-lactamases among *K. pneumoniae* ertapenem resistant isolates collected in 2013 in patients admitted at the University Hospital of Coimbra, Portugal. Our working hypothesis was that ertapenem resistance was mediated by KPC-carbapenemase whose determinant could be inserted in a conjugative plasmid, contributing for its spread.

2. MATERIAL AND METHODS

2.1. Clinical Isolates

In the present study, a collection of 27 ertapenen resistant *K. pneumoniae* isolates recovered from patients admitted to the University Hospital of Coimbra from January to December in 2013 was phenotypic and genetically characterized. The study was conducted at the Microbiology Laboratory of the Faculty of Pharmacy, University of Coimbra, Portugal.

2.2. Antimicrobial Susceptibility Testing and Phenotypic Detection of β-Lactamases

All isolates were resistant to ertapenem according to the antimicrobial susceptibility results obtained from the automated system (Vitek 2 Avanced Expert System-AES, BioMérieux, Portugal) used by the hospital.

The Kirby-Bauer disc diffusion standard method was used for antimicrobial susceptibility testing of selected antibiotics and for phenotypic screening of some β-lactamases. Briefly, a bacterial suspension with a turbidity similar to 0.5 McFarland standard was streaked on Mueller-Hinton agar plates (OXOID) followed by placement of antibiotic disks and incubation during 18-24 hours at 37^{o}C. All isolates were tested for amoxicillin plus clavulanic acid (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), aztreonam (30 µg), imipenem (10 µg) and ertapenem (10 µg) (OXOID). Interpretation was performed according to the EUCAST breakpoint guidelines [62].

Phenotypic confirmation of ESBLs was performed by using the double disk synergy test (DDST) with ceftazidime and cefotaxime placed 15 mm from centered amoxicillin plus clavulanic acid disk diffusion [48]. An increased inhibition halo or ghost inhibition zone for ceftazidime or cefotaxime is considered as a positive test for ESBL.

Detection of inducible AmpC was carried out by disc approximation test using imipenem, cefoxitin and clavulanic acid as inductors agents [63] against ceftazidime or cefotaxime [66]

with 15 mm separation distance. A zone distortion (D shape) for cefotaxime or ceftazidime near to cefoxitin, imipenem or amoxicillin-clavulanic disk is considered as positive test for inducible AmpC β-lactamase.

2.3. Molecular Detection and Identification of β-Lactamases

PCR based methodology was applied for all isolates as confirmatory method for detection of the most common ESBLs (TEM, SHV and CTX-M) [6] and for the most important mechanism for carbapenem resistance in *Enterobacteriaceae* (*Klebsiella pneumoniae* carbapenemase-KPC) [19], while DHA-1 was detected only for positive inducible AmpC producing *K. pneumoniae* isolates by the antagonism test [15, 96].

DNA extraction was performed using the fast extraction methodology with a fresh bacterial cell culture. An 18-24 hours bacterial loop full was suspended into 500 µl of Milli-Q water in a sterile 1.5 ml Eppendorf tube, then homogenized and centrifuged at 10000 rpm during 3 minutes.

Using a pipette, the supernatant was carefully removed and the pellet homogenized with 150 µl of Milli-Q water and boiled at 100^{o}C during 15 minutes for bacterial cell lyses using a dry bath.

After the incubation period, bacterial lysates were submitted to a centrifugation at 10000 rpm during 5 minutes. The supernatant was then removed to a new Eppendorf tube and stored at -20^{o}C and used as DNA template for all assays.

PCR for detection of β-lactamases genes was performed in a 20 µl reaction mixture containing 10 µl of master mix 2X Dynazyme F-508 (Finnzymes), 8 µl of distilled water, 0.5 µl (10 µM) of each primer and 1 µl of DNA template. One positive control and one negative control (water) were included in each amplification reaction. All PCR amplifications were performed on the BioRad Thermo Cycler MJ Mini, either as simplex PCR (for KPC, CTX-M and DHA-1 genes) or duplex PCR for TEM and SHV β-lactamases.

The amplification reaction for KPC gene was: 94°C for 10 minutes, followed by 30 cycles of 94°C for 45 seconds, 62°C for 45 seconds and 72°C for 90 seconds and a final extension cycle at 72°C for 10 minutes. The detection protocol for TEM, SHV and CTX-M was 94°C for 5 minutes, followed by 29 cycles of 94°C for 1 minute, 56°C for 1 minute and 72°C for 1 minute and one final cycle at 72°C for 10 minutes. Finally, DHA-1 detection was performed with initial heat step at 94°C for 3 minutes, followed by 34 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute and one final cycle at 72°C for 7 minutes. The amplified products were separated in 1% of agarose gel electrophoresis and then visualized under UV light after staining with ethidium bromide. Primers sequences and amplicon size for TEM, SHV, CTX, KPC and DHA-1 β-lactamases are shown in the Appendix 1.

Sequencing of selected β-lactamases genes was performed at STABVIDA, Caparica, Portugal, after amplicon purification. The purification procedure was done by mixing 10 µl of the amplified PCR product and 4 µl of ExoSAP-IT (USB Corporation, USA) into 0.2 ml PCR tubes and incubated at 37°C for 15 minutes, followed by enzyme inactivation step at 80°C for 15 minutes.

The online BLAST nucleotide sequence software was used to query β-lactamases sequences for identification of their genetic variants.

2.4. Plasmid Conjugation Assays

Plasmid conjugation assays were conducted to verify the potential of transfer of antibiotic resistance carrying genes. Isolates of *K. pneumoniae* carrying KPC, CTX-M and DHA-1 β-lactamases were used as donor strains, while the sodium azide resistant variant *E. coli* J53 was used as the recipient.

Transconjugants were selected in Trypticase Soy Agar (TSA) plates prepared with 100 µg/ml of sodium azide and 1.25 µg/ml of cefotaxime.

Briefly, in the first day of the assay, selected isolates and the receptor strain, *E. coli* J53 were streaked out on TSA plates and incubated overnight at 37^{o}C to get fresh cultures.

Then, one colony of the bacterial strain was inoculated into assay tubes containing 5 ml of Trypticase Soy Broth (TSB) and incubated at 37^{o}C in a strong shaking water bath during 3 hours.

After the incubation period, 1 ml of the receptor strain *E. coli* J53 was mixed with 100 µl of the donor strain into new tubes containing 5 ml of the TSB medium and incubated 18-24 hours at 37^{o}C in a slight shaking water bath.

After that, 500 µl of the bacterial culture were removed and centrifuged at 10000 rpm for 10 minutes and the supernatant discarded.

The pellet was suspended into 50 µl of PBS buffer and 1:1000 dilution was done. Then, 20 µl of the bacterial solution was plated into TSA selection plates with sodium azide (100 µg/ml) and cefotaxime (1.25 µg/ml) and incubated 18-24 hours at 37^{o}C.

After incubation, two bacterial colonies were isolated and plated into new separate selection media plates and incubated 18-24 hours at 37^{o}C, and then stored into TSB with 15% glycerol for further analysis.

Characterization of the transconjugants was done by performing antimicrobial susceptibility and DNA extraction followed by PCR for detection of specific β-lactamases.

2.5. Molecular Typing of Bacterial Isolates

Plasmid typing was performed to characterize the most common Enterobacteria antibiotic resistance carrying plasmids [89], while assessment of clonal relatedness was carried out by PFGE [79] and MLST [97].

2.5.1. Plasmid Typing

The PBRT protocol was performed for all isolates to identify the most common plasmid Inc groups occurring among *Enterobacteriaceae*, namely, FIA, FIB, FIC, HI1, HI2, I1-Iɣ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA [89]. Primers sequences and amplicon size are shown in the Appendix 2.

All PCR assays were performed in a total reaction volume of 20 µl containing 10 µl of master mix 2X Dynazyme F-508 (Finnzymes), 0.5 µl of each primer (10 µM), water volume adjusted accordingly and 1 µl of DNA template. The PCR reaction was performed with an initial step at 94^{o}C for 5 minutes, followed by 30 cycles of denaturation at 94^{o}C for 1 minute, annealing at 60^{o}C with exception for IncF replicon (52^{o}C) for 30 seconds and elongation at 72^{o}C for 1 minute, and a final extension step at 72^{o}C for 5 minutes.

Finally, PCR products were separated by running a 1% agarose gel and visualized through UV light after staining with ethidium bromide.

2.5.2. Plasmid Extraction

Plasmid extraction from the whole bacterial cells was performed for KPC-producing *K. pneumoniae* using both the QIAGEN plasmid Mid purification kit, following the instruction from the manufacture (www.qiagen.com) and the house made methodology proposed by Kado and Liu [93].

Briefly, for the Kado & Liu methodology, bacterial cells were grown overnight in 3 ml of TSA broth media until an optic density of 0.8 at 600 nm. The pellet obtained by centrifugation (5,700 rpm, 4^{o}C, for 7 minutes) was suspended in 1 ml of Tris-Acetate-EDTA (TAE) buffer (40mM of Tris-Acetate, 2mM of Sodium EDTA, pH adjusted to 7.9) followed by the addition of 2 ml of lysing solution (3% SDS and 50 mM Tris (pH 12.6) and mixing by brief agitation.

The solution was incubated at 50^{o}C in a water bath for 20 minutes and then added two volumes of phenol-chloroform solution.

The solution was emulsified by shaking briefly, followed by centrifugation at 6,000 rpm, for 15 minutes at 4°C.

Avoiding the precipitate at the interface, the upper aqueous phase was transferred to new tubes and used for electrophoresis.

Plasmid DNA was separated by running a 0.7% of agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide.

2.5.3. PFGE

PFGE analysis of *Xba*I-digested fresh genomic DNA was based on the PulseNet one day standardized PFGE protocol for *Escherichia coli* O157:H7, *Salmonella* and *Shigella* [98]. Here, 16 KPC positive (out of 18) and four non-KPC isolates from different hospital wards and collection dates were included to study the genetic relationship between KPC and non-KPC isolates. The restriction PFGE patterns were interpreted according to the Tenover *et al*. guidelines [81].

An overnight bacterial culture was suspended in 2 ml of cell suspension buffer (100 mM Tris: 100 mM EDTA, pH 8.0) and adjusted to McFarland density of 3.8-4.2 units.

An aliquot of 400 µl of the adjusted cell suspension was transferred to a 1.5 ml centrifuge tube and 20 µl of proteinase K (20 mg/ml stock, BIORON GmbH, Germany) added and mixed gently with the pipette tip.

Then, an agarose gel Seakem Gold 1% (Lonza, Rockland, ME USA) was prepared in a TE buffer (10 mM Tris:1 mM EDTA, pH 8.0) and maintained at 55^{o}C in a water bath for preparation of gel plugs.

Plugs were made by adding an equal volume (400 µl) of molten 1% SeaKem Gold agarose to each cell suspension, gently mixing by pipetting up and down several times and the mixture immediately dispensed into the wells of a reusable plug mold (Bio-Rad Laboratories, Hercules, CA, USA) and allowed to solidify for 10 minutes at room temperature.

The plugs were transferred into 50 mL polypropylene tubes containing 5 ml of cell lysis buffer (50 mM Tris, 50 mM EDTA (pH 8.0), 1% sarcosine, and 25 µl of proteinase K (20mg/ml stock solution). Cell lysis was performed for 2 h at 54°C in a water bath with vigorous agitation.

After the incubation period, the cell lysis buffer was removed and the plugs were washed two times with 15 ml of sterile Milli-Q water, and four times with 15 ml of TE buffer (10 mM Tris:1

mM EDTA, pH 8.0). Each washing step was performed at 50°C for 15 min with constant agitation and with pre-heated sterile water or TE buffer accordingly.

Following the final wash, the TE was removed and replaced with 5 ml of fresh TE buffer and kept in the fridge until use.

Slices of *K. pneumoniae* plugs were digested with the *Xba*I (NEW England Biolabs) restriction enzyme in a total volume of 200 µl. The plugs were first incubated in a restriction buffer containing 20 µl of NEBuffer4 10X and 180 µl of distilled sterile water per sample and incubated at 37^{o}C for 10 minutes. Then, the restriction buffer was removed and replaced with 200 µl of 173 µl of sterile water, 20 µl of NEBuffer2 10X, 2µl of BSA 100X and 5µl of *Xba*I (50U) per sample and incubated at 37^{o}C overnight in water bath. The restriction mixture was replaced with 200 µl of 0.5X TBE and the samples kept at 4^{o}C until use.

An agarose gel Seakem LE 1% (Cambrex Bio Science, Rockland, ME USA) was prepared in a volume of 150 ml of TBE buffer 0.5X and bacterial plugs were loaded into the gel wells and sealed with the left remnant melted agarose.

Electrophoresis was run in CHEF-DR III System (Bio-Rad Laboratories, Hercules, CA, USA) in total volume of 3L of 0.5X TBE buffer previously cooled to 11^{o}C in the electrophoresis chamber. The program was 11^{o}C, 6V/cm, 120^{o}, 18.5 hours, switch time of 6 s to 36 s. Finally, the gel was stained with ethidium bromide in 0.5X TBE and visualized under UV light.

2.5.4. MLST

Some isolates studied by PFGE were selected from different hospital wards and collection date to evaluate their clonal relatedness and to compare the sequence types (ST) with those found in other studies. Thus, sequencing of seven housekeeping genes (*rphoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB* and *tonB*) in both senses was performed for six isolates (four with KPC and two non-KPC) using specific primers (Appendix 3) as described on the *K. pneumoniae* MLST website [97].

PCR for each housekeeping gene was performed in a 20 µl reaction mixture containing 10 µl of master mix 2X Dynazyme F-508 (Finnzymes), 8 µl of distilled water, 0.5 µl (10 µM) of each primer and 1 µl of DNA template. Amplification conditions were performed with initial step at 94^{o}C for 2 minutes followed by 30 cycles of 94^{o}C for 1 minute, 50^{o}C for 1 minute except for *gapA* (60^{o}C) and *tonB* (45^{o}C), 72^{o}C for 2 minutes and final extension of 5 minutes at 72^{o}C [97].

The amplified PCR products were purified as described for β-lactamases genes (see page 24), and finally, sequenced at STABVIDA, Caparica, Portugal. Allele sequence numbering and the assignment of sequence types (ST) were performed through the *K. pneumoniae* MLST database (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html).

3. RESULTS

3.1. Characteristics of Bacterial Isolates and Study Population

In the present study, a total number of 27 non-duplicated ertapenem resistant *K. pneumoniae* clinical isolates collected from January to December, 2013, from patients admitted to the University Hospital of Coimbra, were phenotypic and genetically characterized for antimicrobial resistance.

Most of the analyzed isolates were recovered from urine (n=13; 48.1%) and blood (n=7; 25.9%) (Fig. 1), from 11 hospital wards, in which Surgery, Gastroenterology and Emergency Services contributed with the majority of the analyzed *K. pneumoniae* isolates with four (14.8%) each, followed by Renal Transplant and Cardiology units with three (11.1%) (Fig. 2). Patient age was available for 23 (85.2%) patients and ranged from 23 to 82 years with an average of 52 years old, while gender was recorded for 25 (92.6%) patients, being male the dominant gender (n=17; 62.9%) (Table 1).

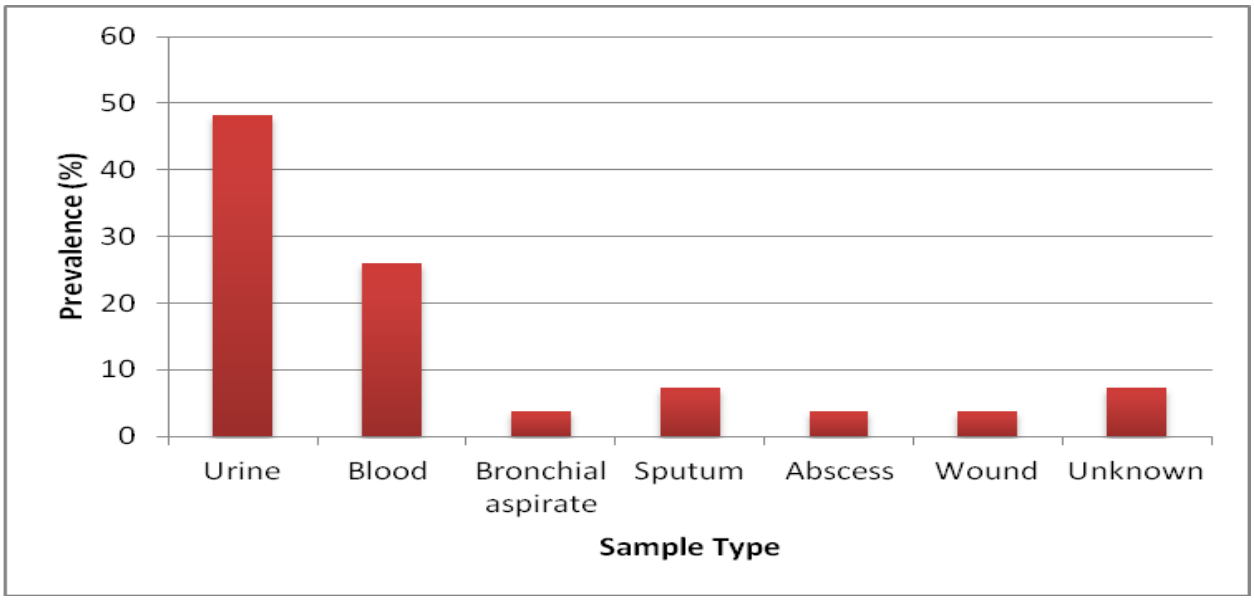

Figure 1. Bacterial clinical sample of isolation for the 27 analyzed *K. pneumoniae* isolates. Urine (n=13), blood (n=7), bronchial aspirate (n=1), sputum (n=2), abscess (n=1), wound (n=1) and unknown (n=2).

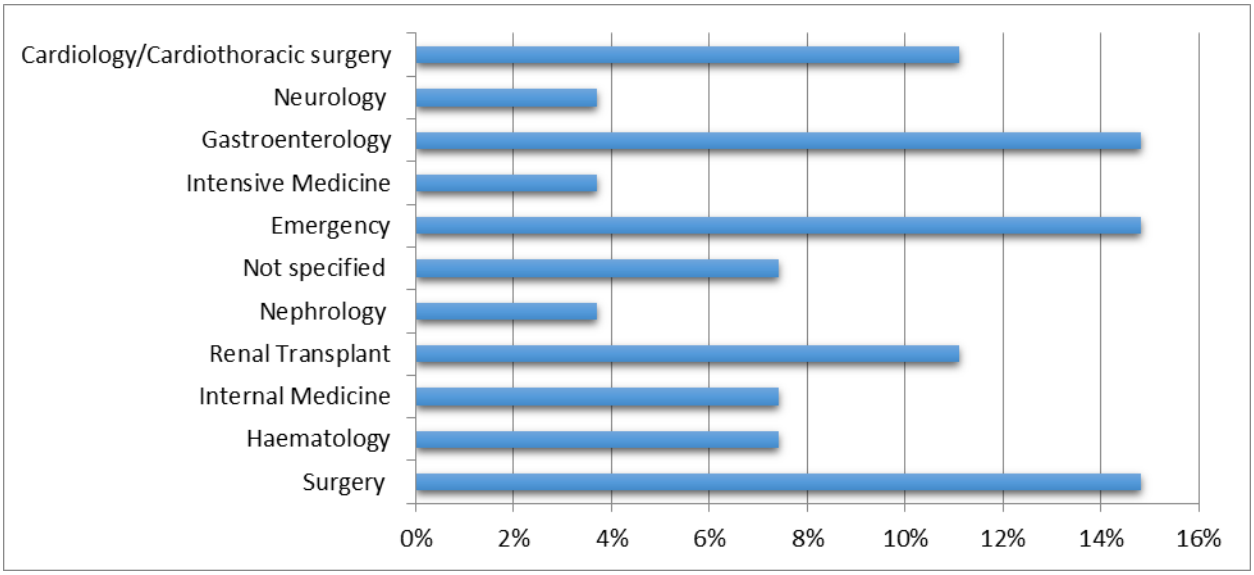

Figure 2. Distribution of the different hospital wards for the 27 analyzed *K. pneumoniae* isolates.

Table 1. Age and gender distribution of the 27 patients with *K. pneumoniae* infection included in the present study.

Age (Years)			**Gender**		
Range	Average	Not specified	Male	Female	Not specified
23-82	52	4(14.81%)	17 (62.96%)	8(29.62%)	2(7.4%)

3.2. Antimicrobial Susceptibility Testing and Phenotypic Detection of β-Lactamases

Antimicrobial susceptibility profile of the *K. pneumoniae* clinical isolates is summarized in the Fig. 3. All isolates were highly resistant almost to all classes of broad spectrum β-lactam antibiotics tested (cephalosporins, monobactams and carbapenems) with an exception to imipenem for which most of the isolates showed only reduced susceptibility (classified as intermediate).

Phenotypic confirmation of ESBLs was detected in 25 (92.6%) of isolates while inducible AmpC β-lactamases was observed in only two (7.4%) (Fig.4). All positive inducible AmpC isolates were detected using either amoxicillin-clavulanic acid or cefoxitin as inductors substrates against cefotaxime and ceftazidime while imipenem induction did not show any antagonistic result (data not showed). A positive result for ESBL and inducible AmpC β-lactamase producing *K. pneumoniae* is shown in the Fig. 5a and 5b.

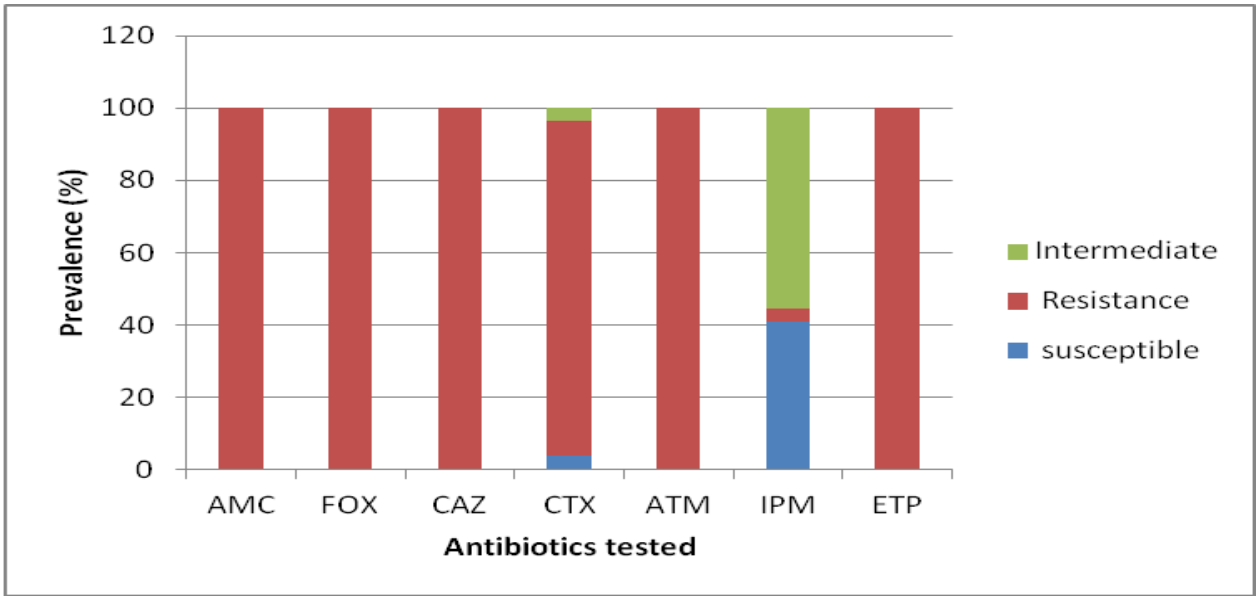

Figure 3. Antibiotic susceptibility profile of the 27 *K. pneumoniae* analyzed isolates. AMC (amoxicillin clavulanic acid), FOX (cefoxitin), CAZ (ceftazidime), CTX (cefotaxime), ATM (aztreonam), IPM (imipenem) and ETP (ertapenem).

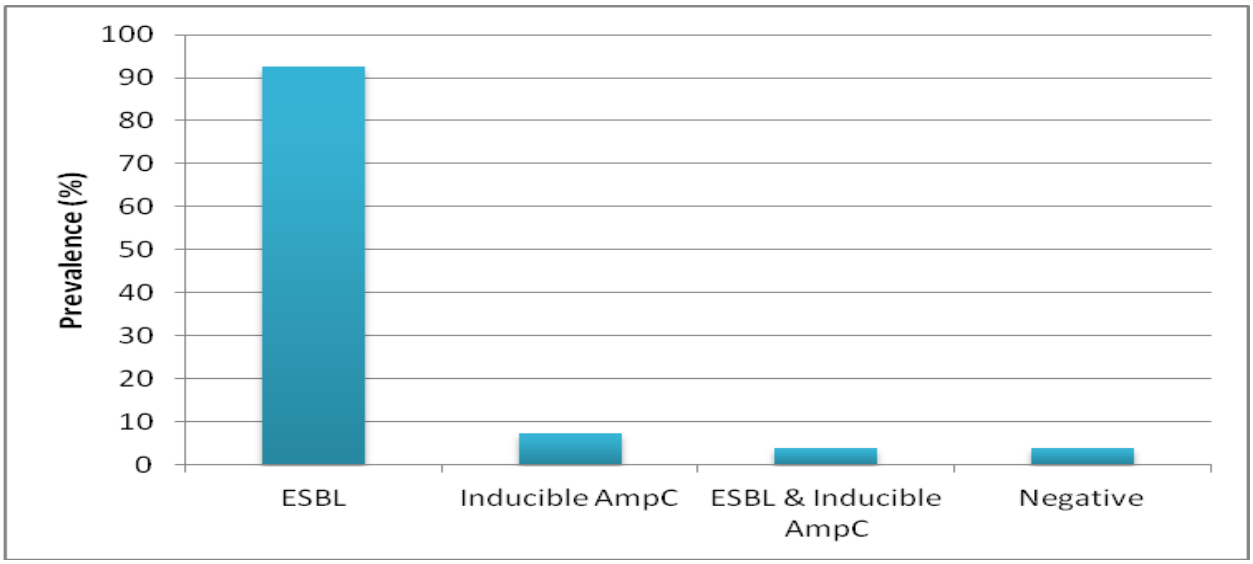

Figure 4. Phenotypic detection of ESBL and inducible AmpC among the 27 *K. pneumoniae* isolates. ESBL (n=25), Inducible AmpC (n=2) and ESBL & Inducible AmpC (n=1).

Figure 5a. Antibiogram showing a positive *K. pneumoniae* producing ESBL. Increased inhibition zone is observed between the central amoxicillin-clavulanic disc (AMC) and cefotaxime (CTX), ceftazidime (CAZ) and aztreonam (ATM) discs.

Figure 5b. Antibiogram showing an inducible AmpC producing *K. pneumoniae*. D-shaped halo is obverved for the antibiotics cefotaxime (CTX), ceftazidime (CAZ) and aztreonam (ATM) surrounding amoxillinin clavulanic (AMC) disc.

3.3. Identification of β-Lactamases Genes

All isolates showed at least one β-lactamase resistance mechanism and the coexistence of different enzymes in the same bacterial isolates was also observed (Fig. 6). A high prevalence of β-lactamases encoded genes was observed with TEM (77.7%), being the most detected enzyme, followed by CTX-M (70.4%) and KPC (66.6%). SHV was detected in 40.7% of the isolates, while inducible DHA-1 was detected in the two AmpC inducible positive isolates (7.4%).

Comparative analysis of ESBLs detection using the phenotypic DDST method and PCR based approaches showed a good concordance (Fig. 7). On the other hand, most of the isolates with reduced susceptibility (14/15; 93.3%) and resistant (n=1; 100%) to imipenem were KPC positive, while only (3/11; 27.2%) of imipenem susceptible *K. pneumoniae* isolates was confirmed as KPC producing (Fig. 8).

The distribution of KPC detection among the study period (January to December, 2013) shows a first outbreak of KPC-producing *K. pneumoniae* in January, and then, from October to December (Fig. 9). Results of DNA sequencing performed in five representative isolates of the outbreak period showed to belong to the KPC-2 genotype variant.

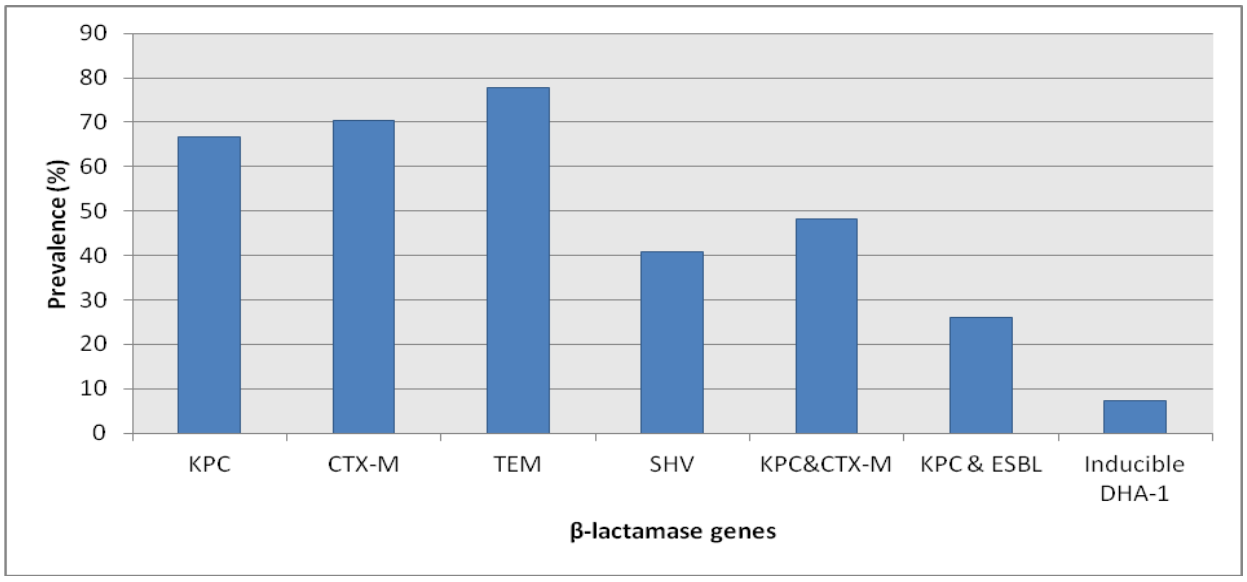

Figure 6. Prevalence of β-lactamases among the 27 *K. pneumoniae* isolates with ertapenem resistance. KPC (n=18), CTX-M (n= 19), TEM (n= 21), SHV (n= 11), DHA-1 (n=2).

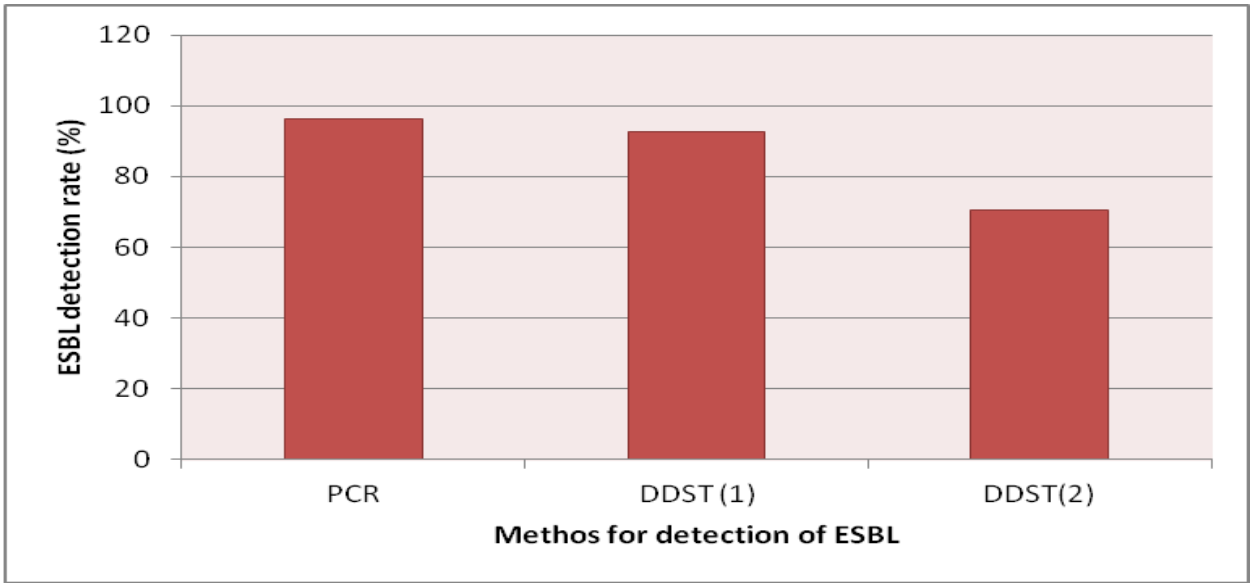

Figure 7. Comparison of ESBLs detection by PCR and using phenotypic DDST method among the 27 analyzed *K. pneumoniae* isolates. DDST (1): using 15 mm separation distance of the discs and DDST (2): using the standard 20 mm disc separation distance.

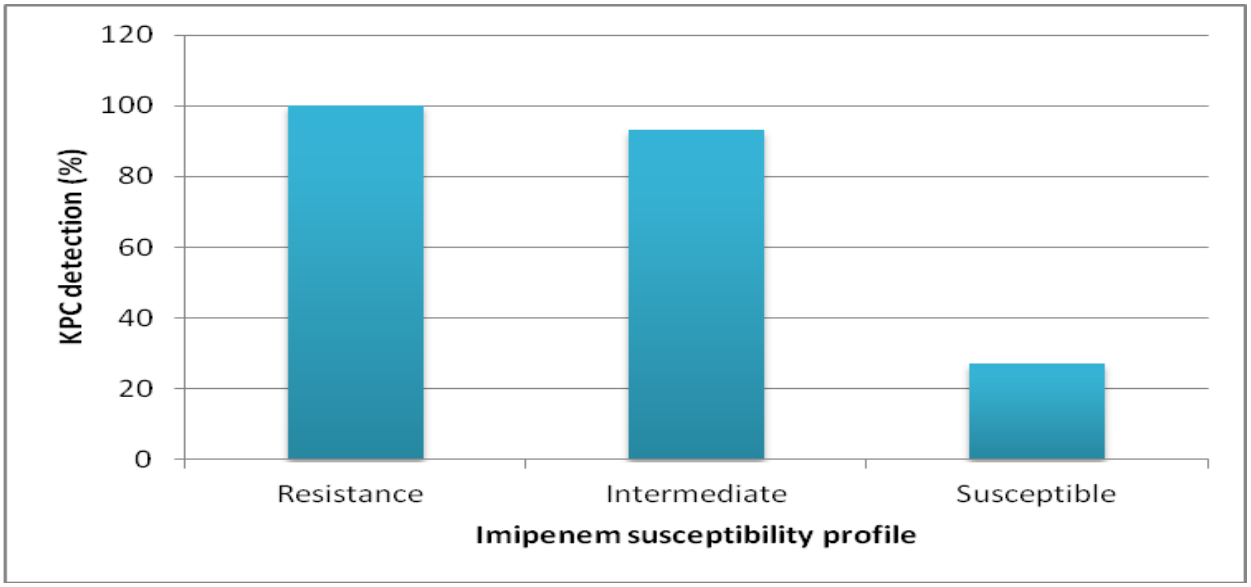

Figure 8. Imipenem susceptibility profile and KPC confirmation by PCR among the 27 analyzed isolates of *K. pneumoniae*. Resistance (n=1), Susceptible (n=11) and Intermediate (n=15).

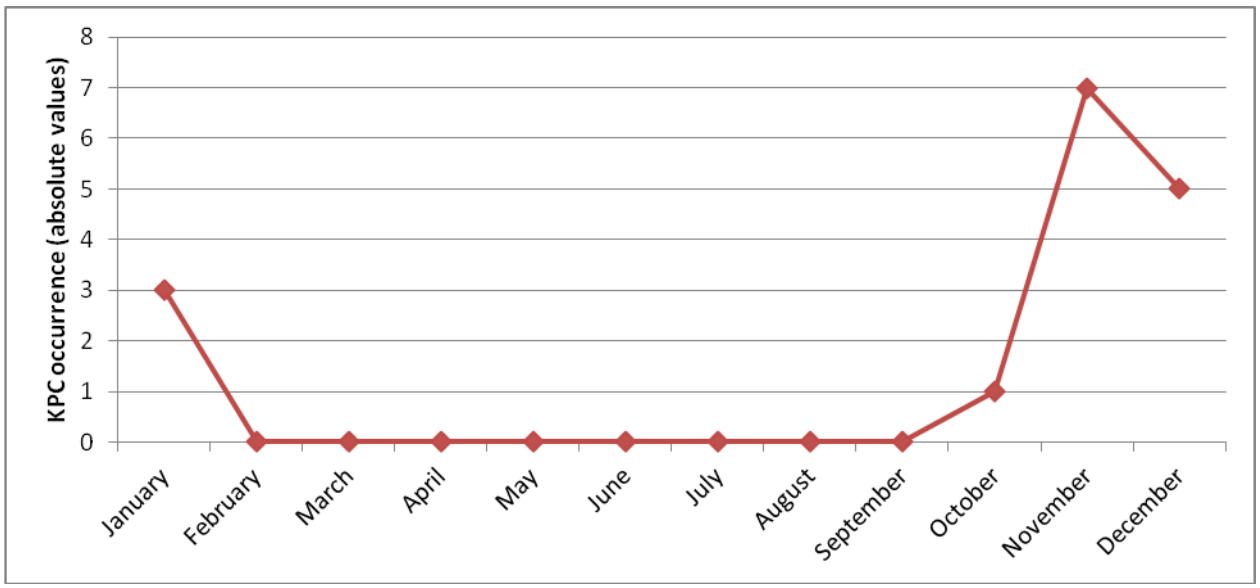

Figure 9. Temporal distribution of KPC for the 27 analyzed *K. pneumoniae* isolates in the Hospital from January to December, 2013. The number of isolates recovered in each period are as following: January (n=3), February (n=1), March (n=1), April (n=0), May (n=1), June (n=1), July (n=0), August (n=4), September (n=1), October (n=1), November (n=7), December (n=5) and two isolates had no collection date record.

3.4. Plasmid Conjugation Assays

Plasmid conjugation assays performed for all KPC carrying *K. pneumoniae* isolates showed a high potential for horizontal transfer of antibiotic resistance genes. A selection plate showing a growth of bacterial culture after conjugation is illustrated in the Fig. 10. Transfer of KPC and TEM β-lactamases was successfully for all *K. pneumoniae* parental isolates, while transfer of CTX-M and SHV was only observed in one isolate. DHA-1 determinant, in contrast, was not transferable to the receptor strain of *E. coli* J53 (Fig. 11). Transferable CTX-M gene was sequenced and confirmed to be the CTX-M-15 determinant.

The Fig. 12 shows a gel image of the KPC amplicons from transconjugants. Analysis of the antibiotic susceptibility profile of the transconjugants is shown in the Fig. 13. The susceptibility profile was generally in concordance when comparing parental isolates and transconjugants, with exception to cefotaxime (CTX) and cefoxitin (FOX) (Fig. 14).

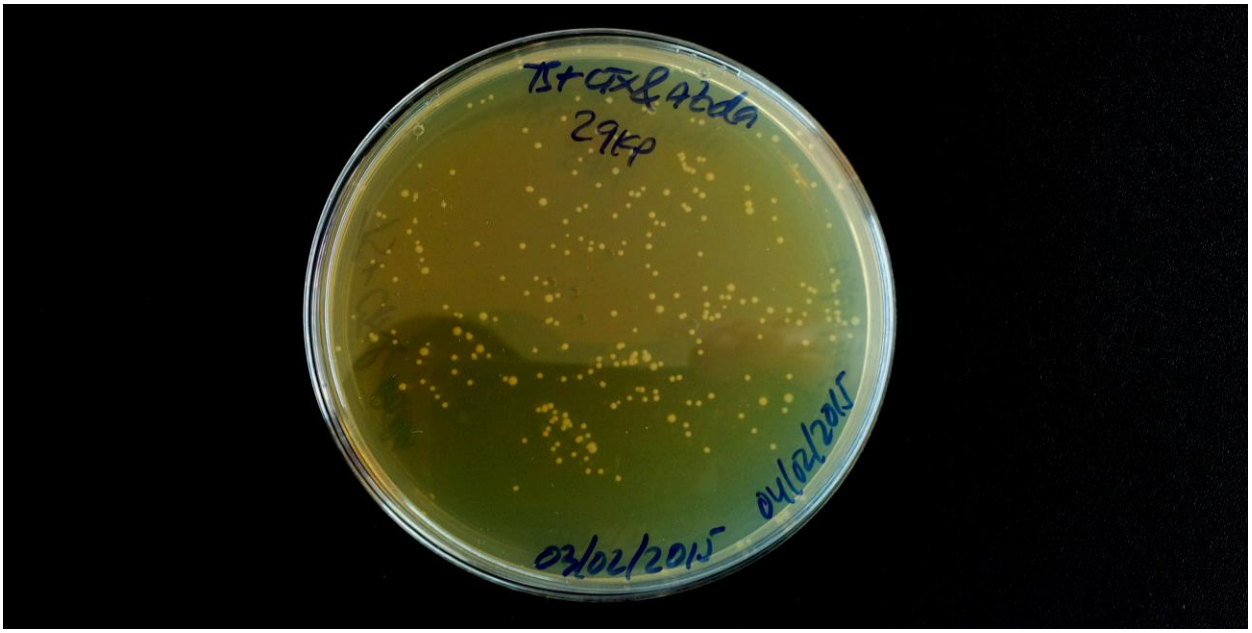

Figure 10. Transconjugants growing in a Trypticase Soy Agar plate supplemented with 1.25µg/ml of cefotaxime and 1mg/ml of sodium azide after 24 hours of incubation.

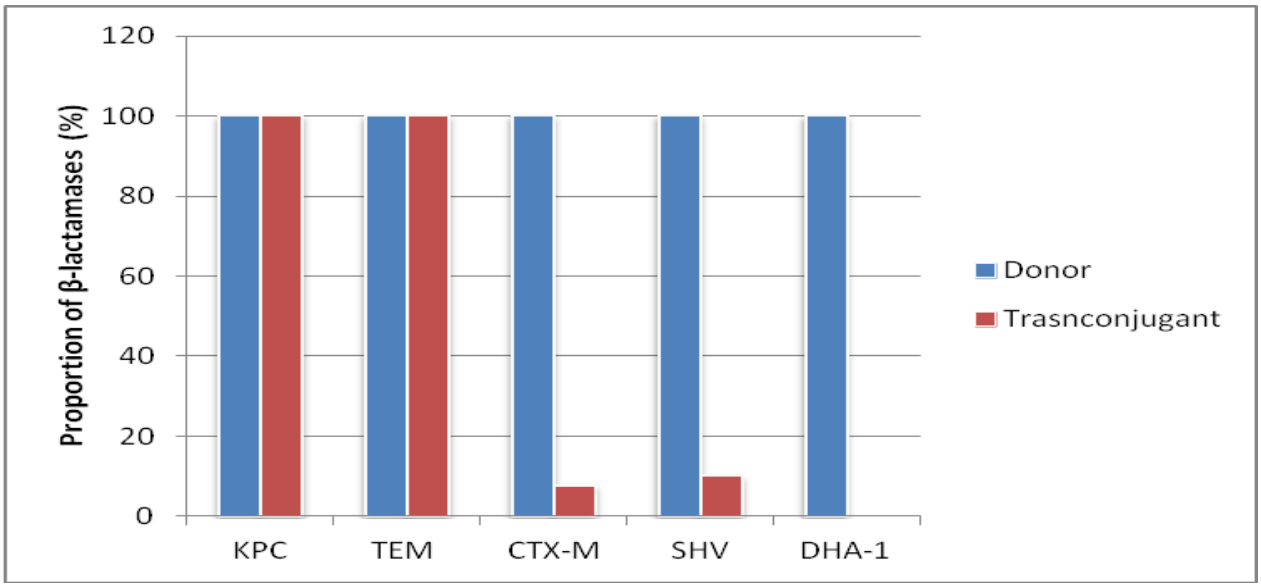

Figure 11. β-lactamases detection among transconjugants. Number of parental isolates: KPC (n=18), TEM (n=15), CTX-M (n=13), SHV (n=10) and DHA-1 (n=2).

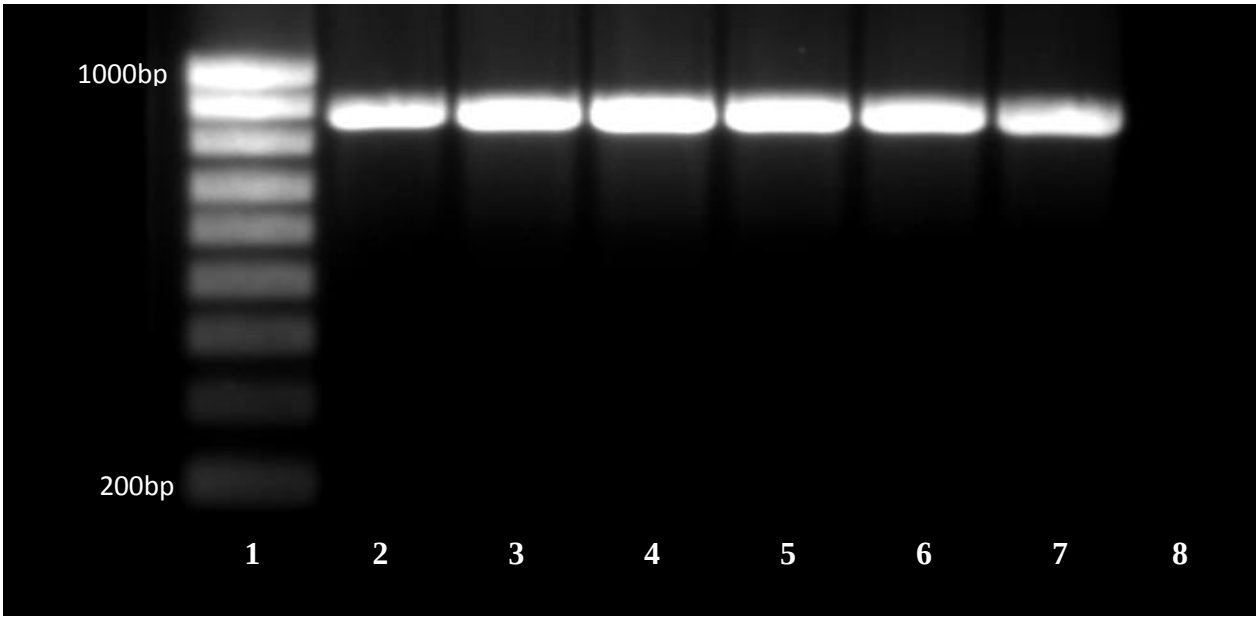

Figure 12. Agarose gel showing isolates of *E. coli* J53 carrying KPC-carbapenemase after conjugation. Lines: 1- molecular marker (1kb, NZYDNA ladder V, nzyTech), 2- positive control (KPC), 3 to 7- transconjugants DNA and 8- negative control.

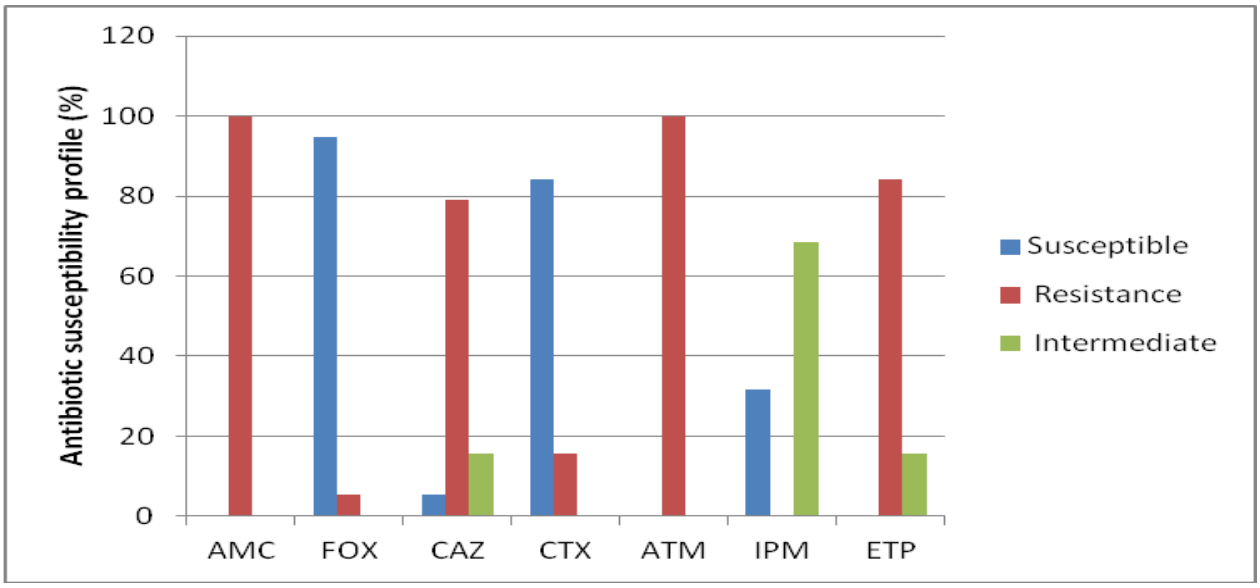

Figure 13. Antibiotic susceptibility profile of transcojugants (n=18 isolates).

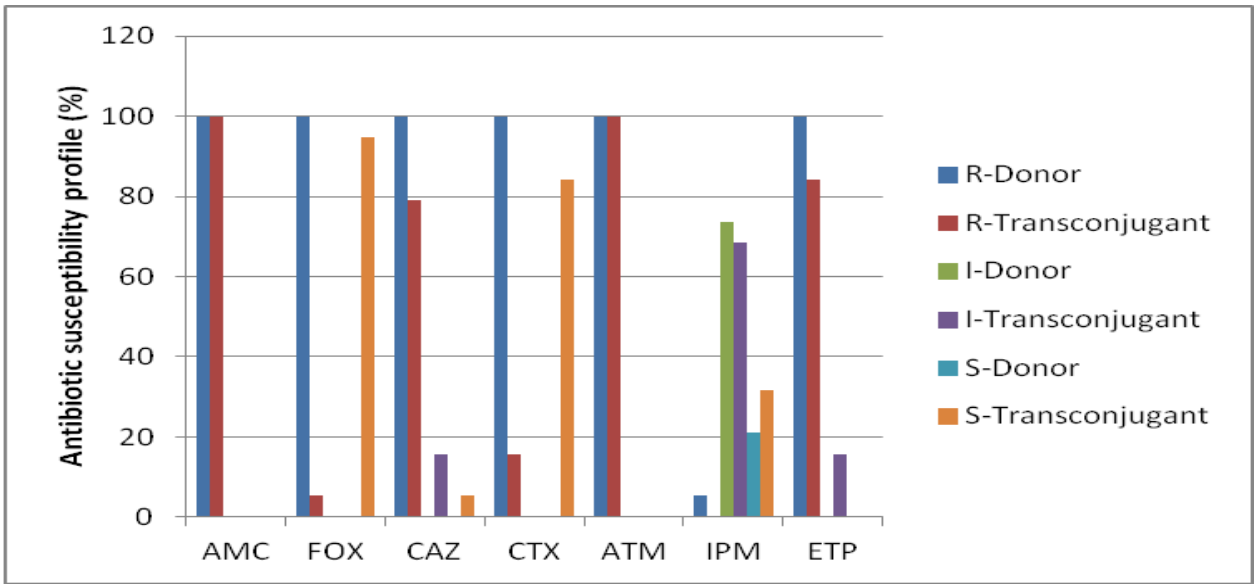

Figure 14. Antibiotic susceptibility profile of donor strains and transconjugants (n=18 isolates). R-resistance, I-intermediate and S-susceptible.

3.5. Molecular Typing of Bacterial Isolates

3.5.1. Plasmid Typing

The PBRT protocol resolved 62.9% of the analyzed *K. pneumoniae* isolates and grouped them into four different plasmid Inc groups (IncF, IncHI1, IncHI2 and IncN), being IncF the mostly found dominant Inc group with 56% (Fig. 15). The coexistence of different plasmids in the same bacterial isolate is shown in the Fig. 16. Plasmid extraction was unsuccessful with both the QIAGEN plasmid Mid purification kit (www.qiagen.com) and the Kado & Liu method [93].

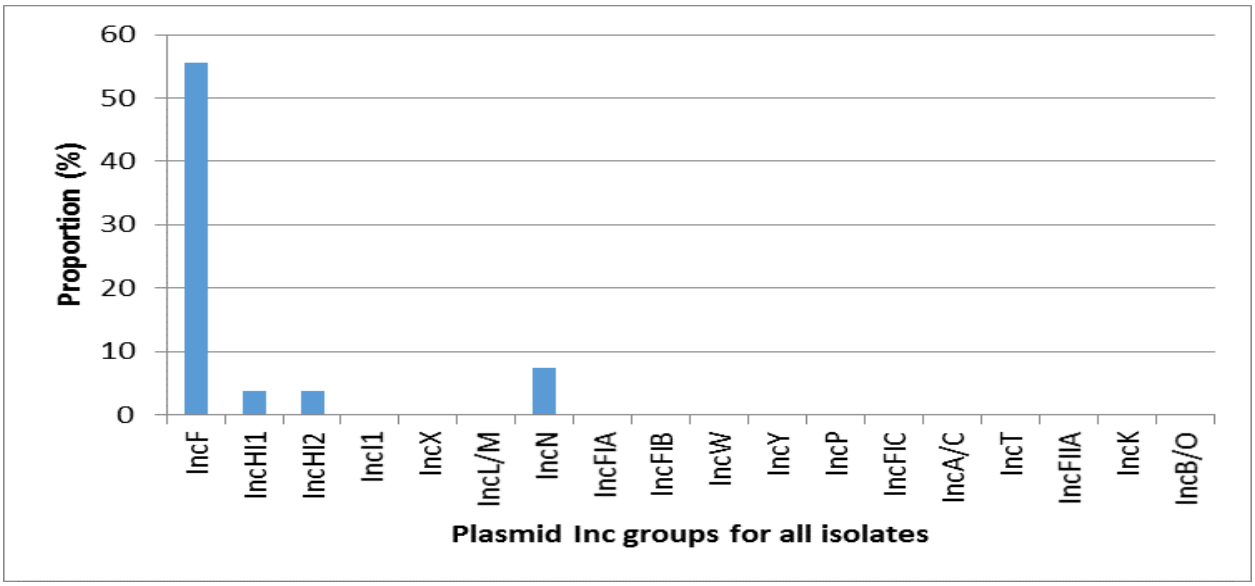

Figure 15. Proportion of plasmid Inc groups among the 27 *K. pneumoniae* isolates. IncF (n=15), IncHI (n=1), IncHI2 (n=1) and IncN (n=2).

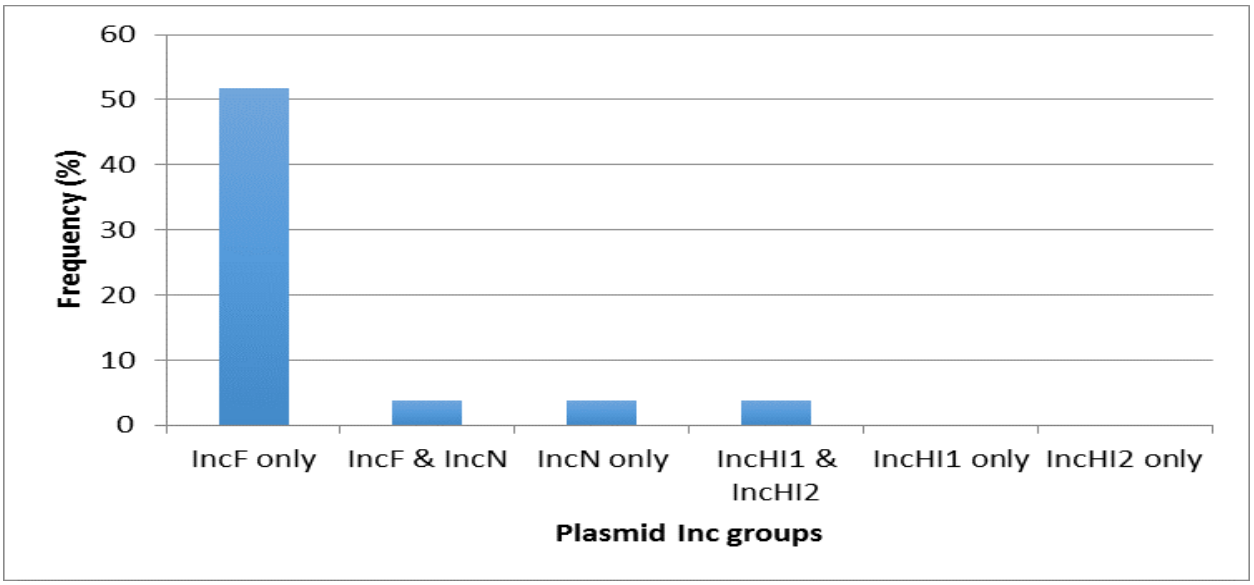

Figure 16. Frequency of coexistence of different plasmids in the same bacterial. IncF only (n=14), IncF & IncN (n=1), IncN only (n=1), IncHI1 & IncHI2 (n=1).

3.5.2. Clonal Relatedness

Analysis of *Xba*I digested genomic DNA for the 16 KPC and four non-KPC isolates showed, seven different PFGE clusters (Fig. 17), according to the Tenover *et al*. algorithm [81]. All KPC-producing isolates belonged to three different PFGE clusters distinct from the non-KPC. Furthermore, the majority of the KPC isolates were represented by one dominant clone with 50% out of 20 total isolates, while all non-KPC isolates were singletons.

MLST analysis of the six isolates (four with KPC and two non KPC) showed three different sequence types (ST), three ST15, two ST348 and one ST11. ST348 was only found in KPC isolates while ST15 was identified in both KPC and non-KPC. ST11 was found in non-KPC isolates. The epidemiological data of KPC and non-KPC producing *K. pneumoniae* with PFGE and MLST analysis is showed in the Table 2. KPC-producing *K. pneumoniae* ST15 was the first sequence type detected at the beginning of the outbreak, in January while ST348 appeared later in October and November, 2013.

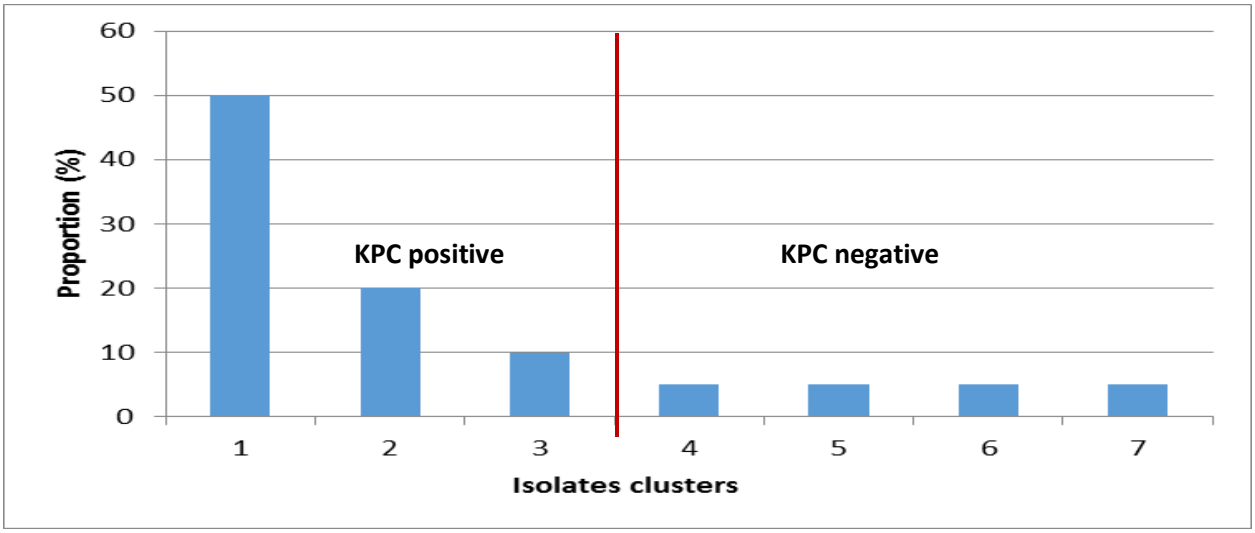

Figure 17. PFGE patterns of KPC and non-KPC producers. Total number of isolates (n=20), with cluster 1 (n=10), cluster 2 (n=4), cluster 3 (n=2) and clusters 4-7 (n=1).

Table 2. Epidemiological data of KPC and non-KPC producing *K. pneumoniae* with PFGE and MLST analysis during the study period in 2013.

Isolate	MLST	PFGE	KPC	Sample	Collection	Hospital Ward
8Kp	ST15	3	Positive	Urine	January	Internal medicine
13Kp	ST15	1	Positive	Urine	December	Emergency
17Kp	ST11	5	Negative	Urine	May	Surgery
26Kp	ST348	1	Positive	Urine	October	Internal medicine
28Kp	ST348	2	Positive	Blood	November	Gastroenterology
39Kp	ST15	7	Negative	Sputum	September	Cardiothoracic surgery

4. DISCUSSION

In the present study, a collection of 27 ertapenem resistant *K. pneumoniae* isolates recovered from equal number of patients admitted to the University Hospital of Coimbra in 2013 was characterized for antimicrobial resistance.

The high proportion of isolates recovered from urine and blood (Fig. 1) is likely to support the clinical importance of *K. pneumoniae* as an important cause of urinary tract and bloodstream infections [2, 99]. On the other hand, the majority of patients included in this study are over 50 years old (Table 1) or underwent surgical procedures (Fig. 2), and thus, making them vulnerable to bacterial infections. It is known that increased age, length of hospital or intensive care unit stay, severity of clinical status, insertion of various types of indwelling catheters, performance of certain types of invasive procedures or surgical interventions, receipt of renal replacement therapy or mechanical ventilation are important risk factors to acquire *K. pneumoniae* infections [100, 101].

The prevalence of antibiotic resistance among the analysed isolates of *K. pneumoniae* is extremely high (Fig. 3). This finding might correlate with the global spreading of antibiotic resistance associated with the extensive use of antibiotics in clinical practices. Third generation cephalosporins such as cefotaxime and ceftazidime, are worldwide used as alternative to treat bacterial infections due to β-lactamase mediated resistance, especially penicillinases, [102] while carbapenems such as imipenem, meropenem, ertapenem and doripenem, are recommended as first line therapy for severe infections due to ESBL producing organisms [16].

The highest resistance to β-lactams observed in the present study correlates with the high production of β-lactamases. All isolates showed at least one β-lactamase resistance mechanism among ESBLs, AmpC and carbapenemases (Fig. 6).

ESBL-producing strains of *K. pneumoniae* are currently found throughout the world, although their phenotypic characteristics and prevalence may vary widely from region to region [9, 103].

In the present study we show a high prevalence of ESBL among clinical isolates of *K. pneumoniae* (Fig. 4).

The DDST method for phenotypic confirmation of ESBL-producing *K. pneumoniae* was successful for 70.3% of the analysed isolates using the standard distance of 20 mm [62]. However, the sensitivity of the test increased to 92.5% when shortening the distance to 15 mm between the centre-to-centre amoxicillin clavulanic and the surrounding discs of cefotaxime and ceftazidime (Fig. 7), suggesting that a reduction of the distance could likely improve the synergism of the DDST, although the 20 mm recommended distance is being used in several studies [61].

Most of ESBLs found were of TEM-type followed by CTX-M- and SHV-type (Fig. 6). TEM and SHV ESBLs are worldwide found among *Enterobacteriaceae* and are derivative of parental TEM-1&2 and SHV-1 which are narrow spectrum enzymes [9]. TEM-1&2 are the most common plasmid mediated β-lactamase associated with ampicillin resistance in Gram-negative, mainly *E. coli*, while SHV-1 is produced by the vast majority of *K. pneumoniae* [9, 102]. Thus, we cannot discard the presence of these penicillinases in the studied isolates however, gene sequencing for TEM and SHV determinants was not determined in the present study.

The high prevalence of CTX-M producing *K. pneumoniae* is correlated with the global dissemination of CTX-M enzymes which is a tremendous problem. Currently, CTX-M-type ESBLs have experienced a global diffusion, outnumbering and partially replacing the TEM- and SHV type ESBLs, and becoming overall the most prevalent type of ESBL [11, 104]. This epidemiological scenario included the increase in the number of different CTX-M enzymes and the recognition of multiple clones and genetic elements carrying CTX-M genes. While TEM and SHV have been associated with epidemic clones, CTX-M in contrast might follow an allodemic pattern, meaning that the increase of CTX-M enzymes had not been only a result of the dissemination of particular clones but of the spread of both multiple specific clones and/or mobile genetic elements [10]. Here, we identified the CTX-M-15 β-lactamase in *K. pneumoniae* isolates, one of the most disseminated and usually associated with the ST131 clone of *E. coli* [11]. Moreover, this enzyme determinant was associated with a conjugative plasmid IncF.

Challenging ESBL detection is the coproduction of AmpC β-lactamses. These enzymes are not inhibited by β-lactamase inhibitors such as clavulanic acid and false reports may occur. Currently, there are no standard guidelines for phenotypic detection of AmpC mediated resistance. Herein, the inducible plasmid DHA-1 was detected in two (7.4%) *K. pneumoniae* isolates, by using amoxicillin-clavulanic acid or cefoxitin as inducers and cefotaxime or ceftazidime as reporter substrates and PCR detection (Fig. 4&6).

Plasmid mediated inducible β-lactamases are rare and they were thought to be non-inducible due to lack of a functional AmpR or absence of an AmpR binding site [105]. However the increasing reports of inducible plasmid DHA-1&2 and ACT-1 have changed the generalization [106], though the mechanism by which AmpC alone or AmpC and AmpR are mobilized from a chromosome into plasmid is still a matter of studies [15]. To our knowledge this is the first report of inducible DHA-1 in clinical isolates of *K. pneumoniae* in Portugal.

On the other hand, treatment of bacterial infections due to ESBL-producing bacteria has been compromised by the emergence of carbapenem resistance due to carbapenemases producing organisms [18]. In the present study, *K. pneumoniae* carbapenemase (KPC) was detected in 66.6% of the analysed isolates of *K. pneumoniae* with ertapenem resistance (Fig. 6).

A part of ertapenem, reduced susceptibility to imipenem has been also suggested as phenotypic marker for carbapenemase mediated resistance. Herein, KPC gene was detected in all isolates with resistance (n=1; 100%) and in 14 (93.3%) with reduced susceptibility to imipenem, and in only three (27.2%) imipenem susceptible isolates (Fig. 8). The present data shows that reduced susceptibility to imipenem associated with ertapenem resistance is likely to be more specific for phenotypic screening of KPC producing *K. pneumoniae*. The high specificity of imipenem or meropenem than ertapenem has been reported [61] and currently imipenem and/or meropenem are recommended for carbapenemase screening. Ertapenem has found to be less specific because isolates with AmpC/ESBL and decreased permeability have higher minimum inhibitory concentrations (MICs) for ertapenem than for imipenem or meropenem [16]. These results also suggest the need of molecular methods to confirm the presence of KPC.

Although KPC has been increasingly reported in different countries, including in Europe [107], their dissemination in Portugal is barely known. Existing data showed an occurrence of KPC-3 in nosocomial isolates of *K. pneumoniae* recovered from hospital wards in Lisbon, in 2012 [35]. Here we report the first KPC-2 carbapenemase mediated resistance in isolates of *K. pneumoniae*, identified at the University Hospital of Coimbra, Portugal, in 2013, which supports their rapid dissemination probably associated with the extensive use of carbapenems.

The global spreading of KPC has been linked to specific clones, being ST258 the most world widely found [20]. However, MLST results performed in selected KPC isolates showed two different sequence types, ST15 and ST348. Moreover, ST15 was also identified in non-KPC isolates together with ST11. Indeed, ST15 *K. pneumoniae* ertapenem resistant lineage was previously reported in this hospital but the resistance to this carbapenem was associated with modifications in the major porins [36] while ST11 and ST348 are novel clones, the latter only reported so far in Tanzania, Southern Africa [108]. KPC-producing *K. pneumoniae* ST15 was first detected at the beginning of the outbreak, in January, while ST348 appeared later, in October and November 2013. It is possible that the previously reported *K. pneumoniae* ertapenem resistant belonging to ST15, acquired a plasmid with the KPC-2 gene, while the novel ST348 clone was imported into the hospital with the KPC-2 or acquired the KPC-2 from ST15.

PFGE analysis revealed three clusters for KPC producing isolates and the majority are represented by only one dominant clone (Fig. 17). Different MLST were found in the same PFGE pattern, or isolates with the same MLST profile, in different PFGE clusters (Table 2). The two methodologies showed less variability within the analysed isolates of *K. pneumoniae*, which was expected since they were collected at the same care facility and within a limited period of time. The MLST data can me comparable among different laboratories from variable geographic locations while PFGE is not exchangeable and may suffer of subjectivity in interpretation. Both methods are not used in routine yet due to the high costs of MLST or time consuming for PFGE.

Of particular importance is the location of β-lactamases genes on plasmids which facilitate their horizontal dissemination. In the present study, plasmid typing showed an occurrence of very limited number of plasmids being IncF the most prevalent (Fig. 15). IncF is the most frequent

plasmid family detected in *Enterobacteriaceae* and has been associated with the dissemination of diverse types of resistance genes including ESBLs such as CTX-M-15 and KPC-carbapenemase [90, 109]. The coexistence of different β-lactamase resistance mechanisms in the same plasmid represents a great concern because may confer selective advantage to the recipient bacterial when several antibiotics are simultaneously administered. Plasmid extraction was unsuccessful with the QIAGEN plasmid Midi purification kit (www.qiagen.com) and the house made methodology proposed by Kado & Liu [93], suggesting that these plasmids, whose presence was confirmed by PCR, might have a high molecular size, which is frequent in *Klebsiella* spp., or a low copy number.

Although the high dissemination of CTX-M has been associated with a high conjugative plasmid-mediated horizontal transfer and clonal spreading [12], herein CTX-M gene was only transferable in one isolate (7.6%) out of 13 CTX-M positive isolates subjected to plasmid conjugation. KPC in contrast was successfully transferred in all isolates analyzed (Fig. 11). The high transmission of KPC correlates with their location on plasmid with high potential for horizontal spreading through conjugation [22]. Herein all KPC were associated with IncF plasmid family.

On the other hand, the low or absence of *in vitro* conjugation of CTX-M might suggest different genetic locations of KPC and CTX-M. IncF carries more than one replicon promoting the initiation of replication [109]. However, the presence of other plasmid types or chromosomal location for CTX-M genes might also be considered. The PBRT protocol used in the present study does not cover all plasmid types occurring among *Enterobacteriaceae* [89]. For example, CTX-M-15 gene was already found in non-self-transferable plasmids or integrated on *K. pneumoniae* chromosome [110].

5. CONCLUSIONS

Antibiotic resistance is a global problem and represents a serious threat for patient care. In the present study we show a high prevalence of β-lactamase mediated antibiotic resistance, including to the most effective carbapenems, among clinical isolates of *K. pneumoniae* in the tertiary care University Hospital in Coimbra, Portugal.

This is the first report of the carbapenemase KPC-2 mediated carbapenem resistance in nosocomial isolates of *K. pneumoniae* in Portugal, which correlates with the global dissemination of KPC enzymes. The high prevalence of KPC-carbapenemase was found associated to a conjugative plasmid with high potential for spreading among other bacterial species.

The fact that most of resistance determinants is represented by only one plasmid family (IncF) is a great concern because might amplify the risk for simultaneously spreading of different resistance genes for different classes of antibiotics, which increases the selection for the recipient bacterial host.

Further studies are needed to investigate the exact genetic location of β-lactamases genes, especially for CTX-M-type, which was not transferred after conjugation.

The majority of the analysed nosocomial isolates of *K. pneumoniae* belong to a few clones represented by three MLST profiles, ST11, ST15 and ST348, the latter only found so far in Tanzania, Southern Africa.

Finally, continuous surveillance of antibiotic resistance within the hospital together with rational use of antibiotics is needed to improve patient management and infection control measures.

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7. APPENDIX

Appendix 1. Primers sequences used for detection of β-lactamases.

Primer	Sequence	Annealing	Amplicon	Reference
TEM-F TEM-R	TACGATACGGGAGGGCTTAC TTCCTGTTTTTGCTCACCCA	56^{o}C	716 bp	Mendonça *et al*., 2007
SHV-F SHV-R	TCAGCGAAAAACACCTTG TCCCGCAGATAAATCACCA		471 bp	
CTX-F CTX-R	TTTGCGATGTGCAGTACCAGTAA CGATATCGTTGGTGGTGCCATA		543 bp	
KPC-F KPC-R	TATCGCCGTCTAGTTCTGCTCTCT ACTGCCCGTTGACGCCCAAT	62^{o}C	871 bp	Present Study
DHA 1-F DHA 1-R	CTGATGAAAAAATCGTTATC TTATTCCAGTGCACTCAAAATA	55^{o}C	1140 bp	

Appendix 2. Primers used for identification of plasmid Inc groups (Carattoli *et al*., 2005).

Primer	Sequence	Amplicon Size (bp)	PCR Type
HI1 FW	5-ggagcgatggattacttcagtac-3	471	Multiplex 1
HI1 RV	5-tgccgtttcacctcgtgagta-3		
HI2 FW	5-tttctcctgagtcacctgttaacac-3	644	
HI2 RV	5-ggctcactaccgttgtcatcct-3		
I1 FW	5-cgaaagccggacggcagaa-3	139	
I1 RV	5-tcgtcgttccgccaagttcgt-3		
X FW	5-aaccttagaggctatttaagttgctgat-3	376	Multiplex 2
X RV	5-tgagagtcaatttttatctcatgttttagc-3		
L/M FW	5-ggatgaaaactatcagcatctgaag-3	786	
L/M RV	5-ctgcaggggcgattctttagg-3		
N FW	5-gtctaacgagcttaccgaag-3	559	
N RV	5-gtttcaactctgccaagttc-3		
FIA FW	5-ccatgctggttctagagaaggtg-3	462	Multiplex 3
FIA RV	5-gtatatccttactggcttccgcag-3		
FIB FW	5-ggagttctgacacacgattttctg-3	702	
FIB RV	5-ctcccgtcgcttcagggcatt-3		
W FW	5-cctaagaacaacaaagccccccg-3	242	
W RV	5-ggtgcgcggcatagaaccgt-3		
Y FW	5-aattcaaacaacactgtgcagcctg-3	765	Multiplex 4
Y RV	5-gcgagaatggacgattacaaaacttt-3		
P FW	5-ctatggccctgcaaacgcgccagaaa-3	534	
P RV	5-tcacgcgccagggcgcagcc-3		
FIC FW	5-gtgaactggcagatgaggaagg-3	262	
FIC RV	5-ttctcctcgtcgccaaactagat-3		
A/C FW	5-gagaaccaaagacaaagacctgga-3	465	Multiplex 5
A/C RV	5-acgacaaacctgaattgcctcctt-3		
T FW	5-ttggcctgtttgtgcctaaaccat-3	750	
T RV	5-cgttgattacacttagctttggac-3		
FII_S FW	5-ctgtcgtaagctgatggc-3	270	
FII_S RV	5-ctctgccacaaacttcagc-3		
$F_{rep}B$ FW	5-tgatcgtttaaggaattttg-3	270	Simplex F
$F_{rep}B$ RV	5-gaagatcagtcacaccatcc-3		
K/B FW	5-gcggtccggaaagccagaaaac-3	160	Simplex K&B/O
K RV	5-tctttcacgagcccgccaaa-3		Simplex K
B/O RV	5-tctgcgttccgccaagttcga-3	159	Simplex B/O

Appendix 3. Primers used for MLST of *K. pneumoniae* (Diancourt *et al*., 2005)**.**

Primer	Sequence	Annealing	Amplicon
rpoB-F	GGCGAAATGGCWGAGAACCA	50^{o}C	501 bp
rpoB-R	GAGTCTTCGAAGTTGTAACC		
gapA-F	TGAAATATGACTCCACTCACGG	60^{o}C	450 bp
gapA-R	CTTCAGAAGCGGCTTTGATGGCTT		
mdh-F	CCCAACTCGCTTCAGGTTCAG	50^{o}C	477 bp
mdh-R	CCGTTTTTCCCCAGCAGCAG		
pgi-F	GAGAAAAACCTGCCTGTACTGCTGGC	50^{o}C	432 bp
pgi-R	CGCGCCACGCTTTATAGCGGTTAAT		
phoE-F	ACCTACCGCAACACCGACTTCTTCGG	50^{o}C	420 bp
phoE-R	TGATCAGAACTGGTAGGTGAT		
infB-1F	CTCGCTGCTGGACTATATTCG	50^{o}C	318 bp
infB-1R	CGCTTTCAGCTCAAGAACTTC		
tonB-1F	CTTTATACCTCGGTACATCAGGTT	45^{o}C	414 bp
tonB-2R	ATTCGCCGGCTGRGCRGAGAG		