**Universidade de Aveiro** Departamento de Biologia
**Ano 2019**

**Ana Rita Gonçalves da Silva**

# EPIDEMIOLOGY OF β-LACTAMASES PRODUCING STRAINS

# EPIDEMIOLOGIA DE ESTIRPES PRODUTORAS DE β-LACTAMASES

**2019**

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Professora Doutora Sónia Cristina das Neves Ferreira, Professora Auxiliar Convidada do Departamento de Ciências Médicas da Universidade de Aveiro, Aveiro e sob a co-orientação do Dr. Elmano José da Cruz Ramalheira, Diretor do Serviço de Patologia Clínica do Centro Hospital do Baixo Vouga, EPE.

**o júri**

presidente

Profª. Doutora Tânia Isabel Sousa Caetano
Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

arguente

Profª Doutora Susana Isabel Elias Alarico
Investigadora do Centro de Neurociência e Biologia Celular da Universidade de Coimbra

orientadora

Profª Doutora Sónia Cristina das Neves Ferreira
Professora Auxiliar Convidada do Departamento de Ciências Médicas da Universidade de Aveiro

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**palavras-chave**

infeções bacterianas, epidemiologia, *bla*$_{KPC}$, cefalosporinases, novas terapias, comunicação científica

**resumo**

As infeções do trato urinário, tecidos moles e intra-abdominal continuam a ser das principais causas de morbidade e mortalidade mundial. O uso excessivo e inadequado dos antimicrobianos tem levado ao aparecimento de diversas estirpes multirresistentes, sendo este um problema de saúde pública. A resistência aos antibióticos e a falta de novos compostos antimicrobianos leva a complicações terapêuticas.

Neste estudo, avaliou-se a epidemiologia de estirpes produtoras de carbapenemases, bem como, a disseminação de AmpCs em isolados causadores de infeções do trato urinário e tecidos moles. Além disso, no sentido de avaliar novos compostos antimicrobianos realizou-se um estudo com o Ceftalozano/Tazobactam contra Enterobacteriaceae e *Pseudomonas aeruginosa* causadoras de infeções intra-abdominais.

Os resultados obtidos mostraram a presença de *bla*$_{KPC}$ em 50 *Klebsiella pneumoniae*, no total de 64 estirpes resistentes aos carbapenemos. Além disso, foram encontradas 155 estirpes Enterobacteriaceae produtoras de AmpC. Relativamente ao Ceftalozano/Tazobactam, este mostrou-se sensível contra quase todos os isolados (46/47).

Ao longo deste estudo, verificou-se, também, que é essencial alarmar e sensibilizar as pessoas para estes problemas recorrendo essencialmente à educação. Como tal, realizou-se uma aula para 14 estudantes em risco de abandono escolar no Agrupamento de Escolas de Oliveirinha. Neste caso, os resultados foram positivos em mais de 70% dos casos.

Sendo assim, este estudo mostra a urgência de monotorização e controle epidemiológico em relação às estirpes multirresistentes. Da mesma forma, é direcionado para mitigar este problema com novas terapias e ideias para prevenir a resistência aos antibióticos baseados na educação.

**keywords**

bacterial infections, epidemiology, *bla*$_{KPC}$, cephalosporinases, new therapies, scientific communication

**abstract**

Urinary tract infections (UTIs), soft tissue infections (SSTIs) and intra-abdominal (IAIs) infections continue to be the most important causes of morbidity and mortality around the world. The excessive and inappropriate use of antimicrobials has led to the emergence of several multi-resistant strains, which is a public health problem. Antibiotic resistance and the lack of new antimicrobial compounds lead to therapeutic complications.

In this study, we evaluated the epidemiology of carbapenemases-producing microorganisms, the dissemination of AmpCs in isolates causing UTIs and SSTIs. In order to evaluate new antimicrobial compounds, a study with ceftolozane/tazobactam against Enterobacteriaceae and *Pseudomonas aeruginosa* causing intra-abdominal infections was realized.

The results showed the presence of *bla*$_{KPC}$ in 50 *Klebsiella pneumoniae* in a total of 64 carbapenem resistant strains. In addition, 155 AmpC-producing Enterobacteriaceae strains were found. Ceftolozane/tazobactam was effective against almost all isolates (46/47).

Throughout this study, it has also been found that it is essential to raise awareness of these problems, through education. As such, a class was held for 14 students at risk of dropping out at the “Agrupamento de Escolas de Oliveirinha”. In this case, the results were positive in more than 70%.

In final, this study shows the urgency of monitoring and epidemiological control in relation to multi-resistant strains. Also, this work addresses new therapy to mitigate this problem and ideas to prevent antibiotic resistance based on education.

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## List of Abbreviations

AmpC - Ampicillin hydrolysing enzymes

AST - Antimicrobial Susceptibility Testing

*bla*$_{AmpC}$ - gene that confer resistant to β-lactamics

*bla*$_{CTX-M}$ - gene that confer resistant to β-lactamics

*bla*$_{GES}$ - gene that confer resistant to β-lactamics

*bla*$_{IMI}$ - gene that confer resistant to β-lactamics

*bla*$_{IMP}$ - gene that confer resistant to β-lactamics

*bla*$_{KPC}$ - gene that confer resistant to β-lactamics

*bla*$_{mcr-1}$ - gene that confer resistant to β-lactamics

*bla*$_{NDM}$ - gene that confer resistant to β-lactamics

*bla*$_{NMC}$ - gene that confer resistant to β-lactamics

*bla*$_{OXA}$ - gene that confer resistant to β-lactamics

*bla*$_{SHV}$ - gene that confer resistant to β-lactamics

*bla*$_{SME}$ - gene that confer resistant to β-lactamics

*bla*$_{TEM}$ - gene that confer resistant to β-lactamics

*bla*$_{VIM}$ - gene that confer resistant to β-lactamics

CA-IAIs - Community-acquired intra-abdominal infections

CDC - Centre for Diseases Control and Prevention

Ceft/Taz - Ceftolozane/Tazobactam

CFU - colony-forming unit

CHBV - Centro Hospitalar do Baixo Vouga

cIAIs - Complicated intra-abdominal infections

CLSI - Clinical and Laboratory Standards Institute guideline

CPOs - Carabapenemase-producing organisms

CRACKLE - Consortium on Resistance Against Carbapenems in *Klebsiella* and other Enterobacteriaceae

CRE - Carbapenem-resistant Enterobacteriaceae

cUTIs - Complicated urinary infections

DNA – Deoxyribonucleic acid

ECDC - European Centre for Diseases Prevention and Control

EMA - European Medicines Agency

ESBLs - Extended-spectrum β-lactamasesESCPM group - *Enterobacter cloacae*, *Enterobacter aerogenes*, *Serratia marcescens*, *Citrobacter freundii*, *Providencia* spp., and *Morganella morgani*

EU/EEA - European Union and European Economic Area

EUCAST - The European Committee on Antimicrobial Susceptibility Testing

FDA - Food and Drug Administration

HA-IAIs - Intra-abdominal infections associated with healthcare

HAIs - Hospital-acquired infections

HIP - Hospital Infante D. Pedro

IAIs - Intra-abdominal infections

ICU - Intensive care unit

IncFIA - one of the plasmids responsible for carrying Tn*4401d* and $bla_{KPC}$

IncX-4 - one of the plasmids responsible for carrying $bla_{mcr-1}$

MDR - Multidrug-resistant

MICs - Minimal Inhibitory Concentrations

MTS - MIC Test Strip

PBPs - Penicillin-Binding Proteins

PCR - Polymerase chain reaction

PDR - Pan-drug-resistant

RNA – Ribonucleic acid

SMART - Study for Monitoring Antimicrobial Resistance Trends

SSTIs - Skin and soft tissues infections

Tn*4401d* - $bla_{KPC}$ located inside this transposon

UTIs - Urinary tract infections

XDR - Extensively drug-resistant

# General Introduction

The present Master's thesis approaches one of the major public health problems around the globe, namely, in the area of microbiology. This problem is related to antibiotic resistance and outcomes that may arise in hospital infections caused by resistant microorganisms. Due to the excessive use of antibiotics, bacteria have developed several mechanisms of resistance, like the production of enzymes, which have the ability to be disseminated throughout the world. In addition, this work also addresses to new therapy to mitigate this problem and ideas to prevent antibiotic resistance based on education.

## 1. Hospital-acquired infections

Hospital-acquired infections (HAIs) are a major problem in public health. Complications during hospitalization may result in late recovery and increased costs. In the European Union and European Economic Area (EU/EEA) occurred more than 2-5 million new cases of each year, which lead to drastic impacts on morbidity and mortality (Chyderiotis et al. 2018). Normally, HAIs are associated with invasive medical devices, surgical procedures or environmental contamination (Peleg and Hooper 2012).

The major lethal infections are related to lower respiratory tract and bloodstream, on the other end, the most common opportunistic infections are those of the urinary tract (Peleg and Hooper 2012; Smirnov and Egbert 2016; Ma and Hughes 2018). Also, skin and soft tissues infections (SSTIs) are common infections and intra-abdominal infections are the second most frequent source of bacteraemia (Dryden 2009; Maseda et al. 2014).

### 1.1. Urinary tract infections

Urinary tract infections (UTIs) risk factors can be medical, behavioural and genetic. The risks factors of UTIs increase with age and structural or functional urinary tract impairments since they prevent the normal flow of urine. With the aging, the management of UTIs becomes more difficult, due to accumulating diseases, impairment of organ function, decreases physiologic reserve, increase of the frequency of reinfection and higher prevalence of generic drugs interaction. Premenopausal women have more UTIs than equivalent aged men. However, in the older population, the incidence in both genders increases and equalizes. Furthermore, medical conditions, such as, history of previous UTIs, diabetes, vaginitis and behaviour, like, sexual activity, use a diaphragm with spermicide and not voiding before/after intercourse are also a risk factor for a new UTI. In addition, genetic structural abnormalities, trauma from

instrumentation, urinary stones or an enlarged prostate cause impairment to the flow of urine and these are also associated with UTIs (Ma and Hughes 2018).

UTIs are classified based on clinical symptoms, laboratory data and microbiological findings. This type of infections is subdivided into two main categories, uncomplicated and complicated, varying in terms of severity and symptomatology (Grabe et al. 2015; Ma and Hughes 2018).

Uncomplicated and acute UTIs include sporadic or recurrent episodes community acquired. Usually, they are acute cystitis (infection on the bladder, lower urinary tract) and acute pyelonephritis (infection of the kidney, upper urinary tract) in healthy individuals. These UTIs are seen mostly in healthy women, without structural and functional abnormalities in the urinary tract. A complicated UTI is an infection associated with structural or functional abnormalities of the genitourinary tract or the presence of an underlying disease, which increase the risk of a more serious outcome (Grabe et al. 2015).

An infection in the upper urinary tract carries a risk of spread to the bloodstream which may eventually lead to sepsis (Smirnov and Egbert 2016).

For optimal urinary tract infections treatment is necessary to know the pathophysiology, susceptibility of microorganisms that cause infection and find appropriate pharmacologic options (Ma and Hughes 2018).

### 1.2. Skin and soft tissues infections

Skin and soft tissues infections (SSTIs) are common, self-limiting and superficial infections to life-threatening diseases.

These infections can be based on anatomical site, clinical severity of local, microbial cause (aetiology), and diagnosis (systemic signs) into uncomplicated or complicated. Soft tissues are underlying the skin and they include fat layers, fascia and muscle (Eron 2003; Dryden 2009).

SSTIs are inflammatory microbial invasions of the epidermis, dermis and subcutaneous tissues (Dryden 2010).

Successful management of SSTIs involves prompt recognition, adequate antibiotic therapy and surgical intervention, if required. For the treatment of these infections are indicated some β-lactams. In polymicrobial infections, the therapy indicated is β-lactam/ β-lactamase inhibitor combinations (Dryden 2010). In addition, tigecycline (minocycline derivative with a broader spectrum of activity) is approved for the treatment of complicated skin and soft-tissue infections (Peleg and Hooper 2012).

### 1.3. Intra-abdominal infections

Intra-abdominal infections (IAIs) are the second frequent most source of bacteraemia (Maseda et al. 2014).

IAIs are subdivided into uncomplicated (e.g. acute diverticulitis and certain forms of acute appendicitis) and complicated infections (Sartelli et al. 2013). These terms are used to differentiate clinical situations and categorize the severity of infections for treatment recommendations (Maseda et al. 2014). Complicated intra-abdominal infections (cIAIs) include a very large variety of infectious processes and are an important cause of infection. There are different sources of infection, such as biliary obstruction, gallbladder, gastroduodenal, appendiceal or small and large bowel perforations. When patients are diagnosed with peritonitis or with sepsis syndromes, mortality rates are particularly high (Eckmann and Solomkin 2015).

Moreover, intra-abdominal infections can be classified into two groups: community-acquired intra-abdominal infections (CA-IAIs) and intra-abdominal infections associated with healthcare (HA-IAIs). CA-IAIs are directly acquired from the community; while HA-IAIs develop in patients hospitalized or residents in health facilities for long period. HA-IAIs are usually associated with higher rates of mortality. This can be explained since these patients have a deficient immune system, leading to major probability of infection by microorganisms and involvement of resistant pathogens is higher (Sartelli et al. 2013; Maseda et al. 2014).

Principal risk factors in IAIs are associated with age (greater than 70 years), immunosuppression, chronic dialysis, a large number of comorbidities, admission in a healthcare, internment of the patients, recent surgical procedure, recent urinary catheterization, use recent of antimicrobial agents (such as β-lactams and quinolones) and recent story of colonization of MDR (Labricciosa et al. 2018).

Control of intra-abdominal infections depends of the patient's characteristics (comorbidity and previous antibiotic treatment), local resistance data, the context (intensive care unit (ICU), hospital ward) and the origin of the infection (acquired in the community, associated with healthcare or nosocomial) (Maseda et al. 2014). Surgery is the most important therapeutic recourse for controlling intra-abdominal infections. When surgical intervention is required, usually 24-hour perioperative prophylaxis is performed. In contrast, patients with uncomplicated IAIs may be treated non-operatively (Sartelli et al. 2013).

## 2. Microorganisms associated with infections

In 2010, the U.S. National Healthcare Safety Network indicated that Gram-negative bacteria are responsible for more than 30% of HAIs and the Enterobacteriaceae family being the most commonly identified group overall (Peleg and Hooper 2012). Enterobacteriaceae are large and diverse family of Gram-negative rods and microorganisms are facultative anaerobic. This family has members free-living and part of the microbiome (lower gastrointestinal tract), actually some of these are adapted strictly to living in humans. This family may cause diseases when they gain access to normally sterile body sites through the help of surface structures, as pili (Smirnov and Egbert 2016).

For example, Gram-negative microorganisms, mainly Enterobacteriaceae, are the most common cause of UTIs and IAIs (Maseda et al. 2014; Ma and Hughes 2018).

The most associated microorganism with SSTIs is *Staphylococcus aureus*, a Gram-positive bacterium that can produce a variety of toxins and may potentiate their virulence, followed by *P. aeruginosa*, *E. coli* and *Enterococcus* spp. (Dryden 2009).

In addition, the *Klebsiella pneumoniae* is the most common microorganisms resistant to carbapenems, since is the most prevalent species producing and carrying a $bla_{\text{KPC,}}$

As mentioned above, *P. aeruginosa* is one of the most prevalent microorganism that causes UTIs and is one of the major nosocomial pathogens worldwide (Sader et al. 2014). *P. aeruginosa* is an aerobic, motile, Gram-negative rod, slimmer and more pale staining than members of the Enterobacteriaceae. The total number of infections caused by *P. aeruginosa* is higher than the other species of Pseudomonas (Smirnov and Egbert 2016). This microorganism represents around 6% of all Gram-negative bacilli isolated from shock septic in ICU patients; and when are established infections are virulent and difficult to treat (Maseda et al. 2014; Smirnov and Egbert 2016).

Usually, affected patients are almost always debilitated or with immune defences compromised. For initiate infection, the *P. aeruginosa* needs to a break in defences of first-line (like a wound) (Smirnov and Egbert 2016). In patients with severe *P. aeruginosa* infection, the prompt initiation of adequate antimicrobial therapy is extremely important. The delay in the initiation of appropriate antimicrobial therapy is associated with increased morbidity and mortality (Sader et al. 2014).

An increase of infections caused by *P. aeruginosa* and Enterobacteriaceae lead to an increase of morbidity and mortality (Mohamed et al. 2018).

## 3. Antimicrobials - β-lactam antimicrobials

In the first half of 20th-century, the discovery and synthetic production of antibiotics, and their subsequent introduction in medicine revolutionized this area, permitting the treatment of once incurable infections. Nowadays, antibiotics are necessary for overall practice of medicine, such as, surgeries, treatment of cancer, organ transplantation and for immunocompromised patients, among others and reduction of the morbidity and mortality (Van Hoek et al. 2011; Khan et al. 2018).

Nowadays, a number of different antimicrobial agents known is large and can be classified based on their mechanisms of action. Antibiotics can inhibit protein synthesis (aminoglycosides, chloramphenicol, macrolides, streptothricin, and tetracycline) or interact with the synthesis of DNA and RNA (quinolones and rifampin). Also, can inhibit or damage synthesis of bacteria cell wall (β-lactams and glycopeptides) or modify energy metabolism of a microbial cell (sulphonamides and trimethoprim) (Van Hoek et al. 2011).

Nonetheless, the β-lactams are the class of antibiotics that are more frequently used in the treatment of infectious diseases (Moya et al. 2009).

One of the first antibiotics discovered was a β-lactam, the penicillin, in 1924.
Over the last years, many new β-lactam antibiotics were developed maintaining a β-lactam ring in their molecular structure (Van Hoek et al. 2011).

The success of β-lactam antibiotics against most of the Gram-negative pathogens depends essentially on the (i) concentration of antibiotic that reaches the periplasmic space; (ii) resistance to the chromosome-encoded AmpC β-lactamases and extended-spectrum β-lactamases (ESBLs) and (iii) the affinity against main targets of the β-lactam antibiotics, such as penicillin-binding proteins (PBPs) (Moyá et al. 2010; Thomson 2010). The concentration reached in the periplasmic space depends on the balance between diffusion through the outer membrane and expulsion by the efflux pumps. The resistance of AmpC is dependent on whether it does or does not prevent the induction of β-lactamases (Moyá et al. 2010). β-lactams act similarly by binding and inactivating the PBPs, which are proteins involved in biosynthesis and remodelling of peptidoglycan structure in the bacterial cell wall. Failure in PBPs results in inhibition of cell wall synthesis and consequent cell death (Llarrull et al. 2010; Eckmann and Solomkin 2015).

This family includes penicillins and derivates, cephalosporins, carbapenems, monobactams and β-lactams inhibitors (Figure 1) (Van Hoek et al. 2011).

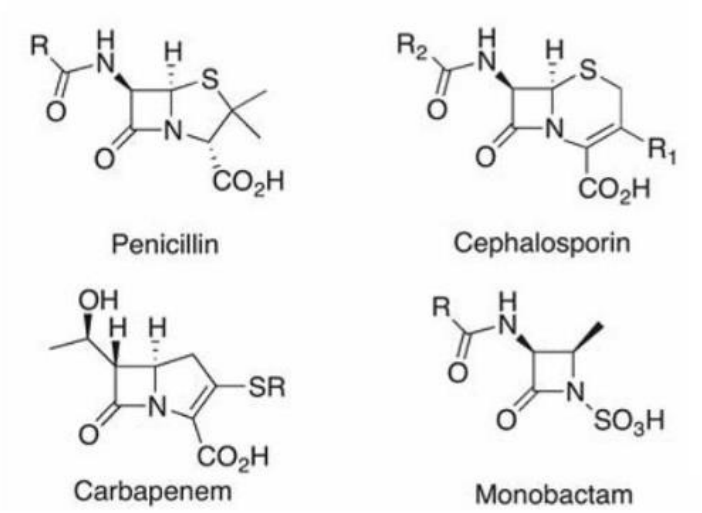


*Figure 1 - Structures of the different β-lactam antibiotic classes (Van Hoek et al. 2011)*

### 3.1. Cephalosporins

Despite the cephalosporins are thought to be a new derivate of penicillin, in fact, they are naturally occurring substances. According to a different spectrum of activity and timing of the agent's introduction, can be differentiated cephalosporins into four groups (first, second, third and fourth generation) (Van Hoek et al. 2011).

In general, first-generation of cephalosporins have good activity against Gram-positive and relatively modest coverage for Gram-negative microorganisms; second-generation of cephalosporins have increased Gram-negative and somewhat less Gram-positive activity; third generation of cephalosporins have improved Gram-negative activity and variable Gram-positive activity; fourth generation of cephalosporins have broad-spectrum activity against both Gram-negatives and Gram-positives microorganisms (Van Hoek et al. 2011).

Also, there are some cephalosporins that show antipseudomonal activity, such as ceftazidime and cefotaxime, since they bind preferentially to PBP3 (present in *P. aeruginosa*), leading to formation of filaments (Moyá et al. 2010).

### 3.2. Carbapenems

Carbapenems are widely used because they are a sub-class of β-lactam antibiotics with a wide range of antibacterial activity (Bina et al. 2015). In addition, carbapenems were recommended for the treatment of infections caused by MDR microorganisms (Mohamed et al. 2018). Carbapenems, like imipenem, bind to PBP2 (present in *P. aeruginosa*), which leads to the conversion of rod-shaped cells into spherical cells; and peptidoglycan cross-linking is compromised, preventing the cell wall synthesis, leading to cell lysis (Moyá et al. 2010; Sebaaly et al. 2018).

Furthermore, excessive use of broad-spectrum antibiotics in hospitalized patients has lead to development of MDR strains, which increase mortality, morbidity and rates of antibiotic resistance (Santos et al. 2011; Magalhães et al. 2017; Khan et al. 2018).

### 3.3. Ceftolozane/tazobactam

Ceftolozane/tazobactam (Ceft/Taz) is a new antibiotic resulting from the combination of a novel third generation cephalosporin, similar to ceftazidime, with tazobactam, a β-lactamase inhibitor. This antimicrobial has activity against extended-spectrum β-lactamases (ESBL)-producing Enterobacteriaceae. In addition, Ceft/Taz has stability when in the presence of AmpC β-lactamases and good pseudomonal activity, which makes it stable against the most common resistance mechanisms of *P. aeruginosa* resistant. Recently, the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved Ceft/Taz for the treatment of complicated intra-abdominal infections (cIAI) and complicated urinary infections (cUTI). Therefore, Ceft/Taz is an alternative to carbapenems for the treatment of infections caused by ESBLs microorganisms (Maseda et al. 2014; Giacobbe et al. 2018; Mohamed et al. 2018; Sheu et al. 2018).

## 4. Antimicrobial resistance

The World Health Organization and the United Nations considered antibiotic resistance as one of the most important public health of the 21st-century. According to the Centre for Disease Control and Prevention, more than 700,000 people die around the world due to resistant infections (Khan et al. 2018).

In preceding decade, it was seen an increase in antimicrobial resistance in nosocomial and community pathogens. The microorganisms have several mechanisms that allow them to adapt to stress, such as genetic mutations (acquisition or sharing of genetic material) and the modulation of genetic expression (Khan et al. 2018). This adaptation is conferred by genes, which are located on transferable plasmids and can be transferred between bacteria, between regions and countries and spreading throughout the world (Bina et al. 2015). This genetic plasticity may lead to resistance to several antimicrobials used in clinical practice. This phenomenon is not recent and is widespread in nature; mutations and/or acquisition of genes make microorganisms "intrinsically" resistant. Also, exist pathogens with "acquired resistance" and that is the most concerning issue in clinical settings (Khan et al. 2018).

The medical literature characterizes different standards of resistance found in antimicrobial resistance in health care. A group of international experts (European Centre for Diseases Prevention and Control, ECDC and Centre for Diseases Control and Prevention, CDC) created a standardized worldwide terminology, which describes acquired resistance profiles of all bacteria responsible for healthcare-associated infection and susceptible to multidrug resistance. They established the terms such as multidrug-resistant (MDR), extensively drug-resistant (XDR) and pan-drug-resistant (PDR). MDR was defined as resistant to at least three or more antimicrobial agents. In XDR bacterial isolates remain susceptible to only one or two categories. PDR was defined as non-susceptibility to all agents in all antimicrobial categories (Magiorakos et al. 2011).

The evolution antibiotic resistance mechanisms and the lack of the antimicrobial agents lead to a key problem for clinicians (Bina et al. 2015).

### 4.1. Mechanism of resistance (Gram-negative) – β-lactamases

Infections caused by Gram-negative bacteria, for example, Enterobacteriaceae and *P. aeruginosa*, are a major cause of concern. These organisms are highly efficient in the acquisition of antibiotic resistance mechanisms, such as loss of porins (family of proteins on the outer membrane of Gram-negative bacteria that allow diffusion of the substrate, such as, antibiotics), increased expression of the transmembrane efflux pump, the presence of antibiotic-modifying enzymes, target site mutations, ribosomal mutations or modifications, metabolic bypass mechanisms and mutation in the lipopolysaccharide (Peleg and Hooper 2012). Gram-negative bacteria combine porin selection (or deletion) to minimize β-lactams access to the PBPs, active efflux to facilitate β-lactams removal (Llarrull et al. 2010) (Figure 2).

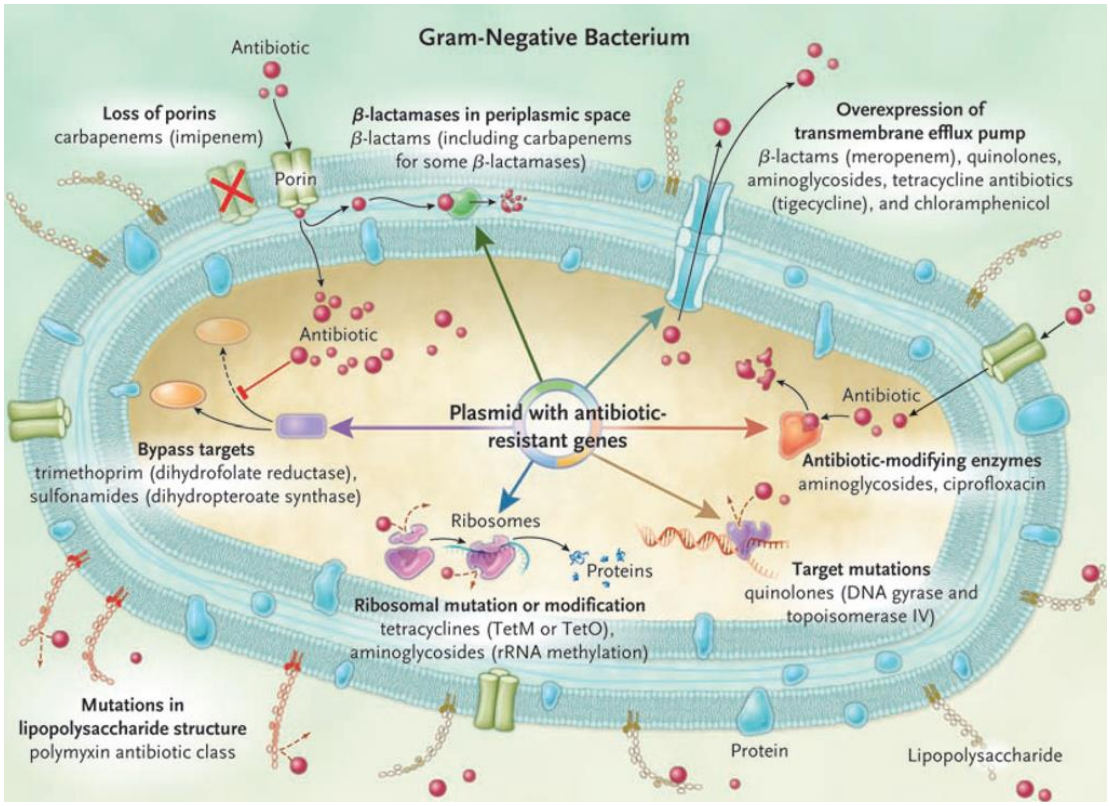


*Figure 2 - Mechanisms of resistance in Gram-negative microorganisms* (Peleg and Hooper 2012)

The generalized commercial use of antibiotics, the diversity, spread, distribution, host diversity and prevalence of β-lactamases activity has increased exponentially. Gram-negative bacteria produce β-lactamases, enzymes which give them resistance to the action of β-lactam antibiotics, since destroy them (Harris 2015) (Figure 3).

The emergence of new β-lactamases confers resistance to the most β-lactam antibiotics, for example, penicillins, carbapenems and cephalosporins (Bina et al. 2015).

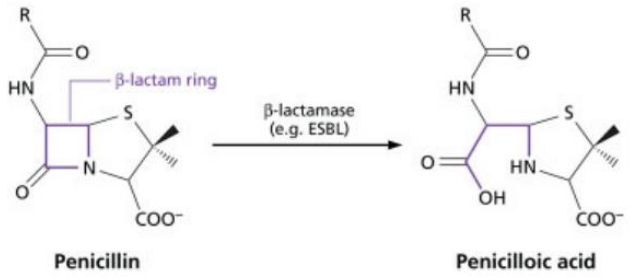


*Figure 3 - Hydrolysis of β-lactam antibiotics by β-lactamases enzymes* (Harris 2015)

β-lactamases can be subdivided into narrow-, moderate-, broad-, and extended-spectrum β-lactamases (ESBLs), based on their activity to hydrolyse a small or considerable number of β-lactams. The broad-spectrum β-lactamases confer resistance to penicillins and cephalosporins; however, inhibitors of β-lactamases, such as clavulanic acid and tazobactam, do not inhibit them. In other hand, the ESBLs are capable to provide resistance to the penicillins, cephalosporins (first-, second-, and third-generation) and aztreonam, but the susceptibility to the carbapenems is maintained and they are inhibited by β-lactamases inhibitors (Van Hoek et al. 2011).

### 4.1.1. Classification of β-lactamases

Over the years, several classification systems for β-lactamases were accepted among researchers. However, only two classification schemes predominated: the classes A to D proposed by Ambler, based on homology of amino acid sequences of β-lactamases type and the Bush-Jacoby–Medeiros functional classification scheme (into four groups (1 to 4) and several subgroups (a to f)). According to Ambler, the division of β-lactamases based on the molecular structure: types A, C, and D with a serine residue at the active site and class B metalloenzymes with a $Zn^{2+}$ cofactor. On other hand, the classification by Bush-Jacoby-Medeiros defines the β-lactamases based on their substrate and inhibitor profiles (Harris 2015).

The first proposal for β-lactamases classification based on the molecular structure was made by Ambler, in 1980, when only four amino acid sequences were known (Bush et al. 1995). In this case, different properties were used to differentiate and classify the β-lactamases, such as: isoelectric point, molecular mass, relative activity towards different β-lactams (‘substrate

profile'), interaction with inhibitors and inactivators, nature of the active site, amino acid sequence, and three-dimensional structure (Brito and Lopes 2016).

The Bush–Jacoby–Medeiros functional classification scheme classifies the β-lactamases according to their substrate and inhibitor profile: (i) cephalosporinases of group 1, are not well inhibited by clavulanic acid; (ii) group 2 penicillinase, cephalosporinases and broad spectrum β-lactamases are generally inhibited by β-lactams directed to the active site; and (iii) the metallo-β-lactamases of group 3, hydrolyse penicillins, cephalosporins and carbapenems, and are also poorly inhibited by almost all molecules containing β-lactams (Bush et al. 1995; Harris 2015).

Increased resistance to β-lactams is reflected in an increase of infections caused by bacteria that expresses ESBLs. In addition, there are bacterial species that possess chromosomally encoded broad-spectrum cephalosporinases (AmpC β-lactamases) (Harris 2015). From the clinical point of view, ESBLs, AmpCs and carbapenemases are the most relevant β-lactamases (Khan et al. 2018).

### 4.2. Extended-Spectrum β-lactamases (ESBLs)

The proliferation of β-lactamases, namely, extended-spectrum β-lactamases (ESBLs) is worrisome (Sader et al. 2014). These enzymes are a big problem because they are capable to provide resistance to penicillins and cephalosporins (first-, second-, and third generation). In addition, these enzymes they have been increasingly described in AmpC producers. The Study for Monitoring Antimicrobial Resistance Trends (SMART) show that 8.5 to 11.2 % of all enterobacterial isolates around the world are ESBL-producing isolates (Van Hoek et al. 2011; Maseda et al. 2014).

Exist some risks factors for ESBLs, such as, the excessive use of third-generation cephalosporins, aminoglycosides and quinolones, previous hospitalization, advanced age, diabetes and use of catheters in ICU patients (Maseda et al. 2014).

There are several and different types of ESBLs, namely CTX-M, TEM, SHV and OXA, encoded by plasmids. CTX-M enzymes are one of the most notable ESBLs since they are disseminated on plasmids in Enterobacteriaceae and subsequently they were rapidly disseminated worldwide (Van Hoek et al. 2011; Weinstein and Logan 2012; Sader et al. 2014; Khan et al. 2018). When microorganisms are hospital-acquired, SHV-type and TEM-type ESBLs are predominant. Nonetheless, the epidemiology of ESBLs is changing, since amino acid

alterations in enzymes give place to extended-spectrum enzymes with broader substrate specificities (Jacoby 2009; Peleg and Hooper 2012).

### 4.3. AmpC β-lactamases

AmpC (ampicillin hydrolysing enzymes) β-lactamases are clinically important cephalosporinases. Nomenclature for AmpCs refers to a family of related enzymes, not to the same protein produced in a variety of microorganisms (Bush et al. 1995; Iredell et al. 2016).

AmpC β-lactamase of *E. coli* was the first bacterial enzyme reported to destroy penicillin named as such in 1940. In Ambler structural classification of β-lactamases, belong to class C; while in the functional classification of Bush–Jacoby–Medeiros, they were assigned to group 1 (Jacoby 2009; Harris 2015). Although these are less common than ESBLs, they have been found in several areas of the world. AmpCs are essentiality isolated from patients after several days of hospitalization, from long-term care facilities, rehabilitation centres and outpatient clinics (Jacoby 2009; Dallenne et al. 2010).

AmpC enzymes can be both chromosomal and plasmid encoded (Maseda et al. 2014).

These enzymes are intrinsic in certain species, such as, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Serratia marcescens*, *Citrobacter freundii*, *Providencia* spp., and *Morganella morganii* – the ESCPM group. Also, non-fermenters bacteria like *P. aeruginosa* has AmpC enzymes with homology to those seen in Enterobacteriaceae (Harris 2015). Chromosomally-encoded expression of AmpC β-lactamases is induced by exposure to β-lactams in many Gram-negative bacteria (Worthington and Melander 2013).

Under β-lactams exposure, AmpCs production is controlled by transcription factors (metabolites of peptidoglycan) and regulators levels. Regulators, like AmpR, respond to changes in cell-wall cycling pathways, leading to strong increases in AmpC levels and expression – inducible expression, present in *E. coli*. In lack of an inducer (absence of antibiotics), AmpR regulator element inhibits $bla_{\text{AmpC}}$ expression as it interacts with peptidoglycan precursors, which repress AmpC expression. When the β-lactams exposition stops, the levels of AmpC usually return to baseline and do not alter the effect of β-lactams. In other words, there are various species have AmpC enzymes with differences in expression levels and clinical significance (Harris 2015; Khan et al. 2018).

Acquisition of *bla*$_{AmpC}$ by plasmids is known since 1989, when they were detected in enterobacteria, which were not expected to produce plasmidic *bla*$_{AmpC}$, like *E. coli* and *K. pneumoniae*. Plasmidic *bla*$_{AmpC}$ mediate resistance to most penicillins (e.g. amoxicillin), first-generation cephalosporins to third generation cephalosporins and β-lactam/ β-lactamase inhibitor combinations. Furthermore, plasmid mediation of *bla*$_{AmpC}$ lead to its dissemination for others organisms within a hospital or geographic region (Alvarez et al. 2004; Jacoby 2009; Harris 2015).

### 4.4. Mechanisms of resistance to carbapenems - Carbapenemases (KPC)

Resistance to carbapenems has become common, representing around 20% of multidrug resistance (Maseda et al. 2014). Resistance to carbapenems can be conferred by a decrease in bacterial outer membrane permeability, through alterations or loss of porins. Deletions or mutations of porin gene difficult this diffusion and is the most common resistance mechanisms in Gram-negative bacteria. In addition, production of cephalosporinases combined with mutations also lead to decrease permeability of the bacterial cell wall. Increased ESBLs and AmpCs production and the expression carbapenemases are also mechanisms of resistance to carbapenems (Weinstein and Logan 2012; Bina et al. 2015; Bonomo et al. 2018).

Carbapenemases are enzymes that hydrolyse carbapenems and are able to break other β-lactam antibiotics, including penicillins, cephalosporins and monobactams (Weinstein and Logan 2012). Production of carbapenemases is the most important mechanism of enzymatic carbapenems resistance in Enterobacteriaceae, since in this family, carbapenemases have high capacity of dissemination and these microorganisms are prone to colonize patients in healthcare settings. The genes of carbapenemases are located in mobile genetic elements, which increases their spread (Bonomo et al. 2018).

The molecular structure classification of carbapenemase based in Ambler belong to class A, B and D. These classes are distinguished by the active site of the hydrolytic mechanism. Class A and D of carbapenemases require serine at their site (known serine carbapenemases), while class B are zinc-dependent, the metallo-β-lactamases. Class A carbapenemases include *K. pneumoniae* carbapenemase (KPC), IMI (imipenem-hydrolyzing enzyme), SME (*Serratia marcescens* enzyme), GES and NMC (not-metallo-enzyme carbapenemase). Most of these enzymes are chromosomally encoded, however *bla*$_{KPC}$ is often carried on a mobile plasmid or transposon. This allows KPC to be the most common enzyme disseminated among

Enterobacteriaceae. On other hand, class D can be able to hydrolyse oxacillin (OXA carbapenemase); have been found more commonly in *P. aeruginosa*, *Acinetobacter* species and in Enterobacteriaceae. In contrast, class B include the plasmid-based Verona integron-encoded MBL (VIM) and IMP; after that New Delhi MBL (NDM) gained much attention because it has spread quickly globally into Enterobacteriaceae species (Weinstein and Logan 2012; Khan et al. 2018).

KPC enzyme is encoded by the *bla*$_{KPC}$, located inside a transposon Tn*4401* which allows it to spread among different species. This transposon is able to enter into various Gram-negative bacterial plasmids. Although *K. pneumoniae* is the most prevalent species producing and carrying a *bla*$_{KPC,}$ this gene has also been identified in another Gram-negative bacillus (Bina et al. 2015). The problem of the production of carbapenemases increases when it's found in Enterobacteriaceae. Since this group of bacteria has a high capacity to disseminate and colonize patients in healthcare settings, which leads to a high concern in the prevention and transmission of these microorganisms (Bonomo et al. 2018).

The prevalence of carbapenemases remains low nonetheless one can not forget that their spectrum of activity encompasses all known β-lactams (from penicillins to carbapenems). In this way, it's necessary to find new alternatives, such as turn once again to combinations of β-lactam antibiotics and β-lactamase inhibitors (Maseda et al. 2014).

## 5. Organization and objectives of the dissertation

The main objectives of this dissertation are: to study antimicrobial resistance, as well as the problems that arise and new alternatives to this problem.

This dissertation is organized into general introduction and four chapters, namely:

1 - In the first chapter of this work, we intend to evaluate the epidemiology of carbapenemases produced by bacteria in the Centro Hospitalar do Baixo Vouga.

2- In the second chapter of this work, the dissemination of AmpC β-lactamases in isolates causing urinary tract infections and soft tissues infectious in the Centro Hospitalar do Baixo Vouga was evaluated.

3 - The third chapter of this dissertation intended to evaluate, *in vitro*, ceftalozane/ tazobactam strips activity against strains of Enterobacteriaceae and *P. aeruginosa* isolated from intra-abdominal infections.

4 - The fourth chapter approaches the global theme present in this dissertation: resistance to antibiotics. In this chapter, pedagogic material was developed and the message passed to the general public.

These chapters reflect the experimental work done and are independent of each other. Each of these chapters is composed of the following sections: introduction, results and discussion and conclusion.

# Material and Methods

## 1. Central Hospital characterization

The present study was performed in Microbiology laboratory of the Clinical Pathology department, in the "Centro Hospitalar do Baixo Vouga, E.P.E. – Hospital Infante D. Pedro (HIP)". HIP is one of the three units that compose the "Centro Hospitalar do Baixo Vouga (CHBV)" and includes several wards, include pathologic anatomical, anesthesiology, cardiology, general surgery, general medicine, dermatology, endocrinology, stomatology, gastroenterology, gynecology/obstetrics and infectiology, among others.

Nowadays, the Clinical Pathology department, include all the main functional areas of clinical-laboratory research and to perform diagnostic tests. This department is composed of the following sectors: general biochemistry, immunology/ autoimmunity and allergology, haematology, laboratory coagulation and microbiology. The mission is to ensure the laboratory tests necessary for laboratory diagnosis lead to increase the level of health protection, that operates 24 hours a day. This department supports hospitalization, external consultation, operating room, outpatient surgery, day hospital, emergency service and provide laboratory support to other health institutions, namely in the region of Aveiro.

Microbiology laboratory realizes tests that contribute to diagnosis, treatment, monitoring and prevention of diseases. Clinical diagnosis makes it possible to detect which microorganism causes an infection and which resistance and/or susceptibility to antimicrobials, being essential for the success of patient treatment.

## 2. Bacterial strains

In the present study, microorganisms were isolated, from both inpatients and outpatients and according to the different objectives of each chapter. The samples were collected between in 2017 and 2018.

In chapter I, carbapenemases-producing bacteria were isolated, from January 2017 to March 2019 with different clinical histories. In this case, it was evaluated the main mechanisms of resistance to carbapenems.

In chapter II, Gram-negative strains producing AmpC β-lactamases, causing urinary tract infections and soft tissues infections were isolated from September to December 2018.

In chapter III, Gram-negative strains from patients with complicated intra-abdominal infections were isolated from September to November 2018. These strains should be extended-spectrum β-lactamases (ESBLs) and/or AmpC β-lactamases producers.

The workflow for each sample followed the laboratory procedures for each biological product. Briefly, samples were inoculated in the appropriate culture medium, and incubated for different hours, depending by local of harvest, at 35 ℃. It should be noted that in order to obtain reliable results, it is necessary that the biological products be harvested correctly. Non-significant, dubious and significant bacteriuria is considered when the count is $<10^3$ CFU/ ml, 10-100 CFU/ ml and $\geq 10^5$ CFU/ ml, respectively.

The strains of interest were placed in hypertonic cryopreservative solution and stored at -20 ℃, in eppendorf, until further use.

Considering the provenance of the patients, the microorganisms were classified as nosocomial and non-nosocomial. If they were collected from patients in the emergency room or external consultation, the microorganisms were considered non-nosocomial. On the other hand, if patients were in the hospital for 72h or more, the microorganism was considered nosocomial.

## 3. Identification and susceptibility testing

Several phenotypic tests are performed in order to achieve a presumptive identification of the bacteria. Identification of the bacterial species and antibiotic susceptibility profile was performed by an automatic detection system, VITEK®2.

### 3.1. VITEK®2

VITEK®2 is an automated system that allows the identification of the bacterial species and antibiotic susceptibility pattern of the microorganism in a 24-hour period, through the inoculation and incubation of cards of identification, created for this purpose. VITEK®2 is configured according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and VITEK®2AES software (Advanced Expert System) (BioMérieux, Marcy L'Étoile, France). The system is based on an optic system combining a multichannel fluorometer and photometer that registers alterations in fluorescence, turbidity and colour.

Identification cards were used for Gram-negative microorganisms (GNI). After this identification, the respective antimicrobial susceptibility cards are selected. In this work, AST-N373 and AST-N355 cards were used for susceptibility to antimicrobials in Gram-negative microorganisms.

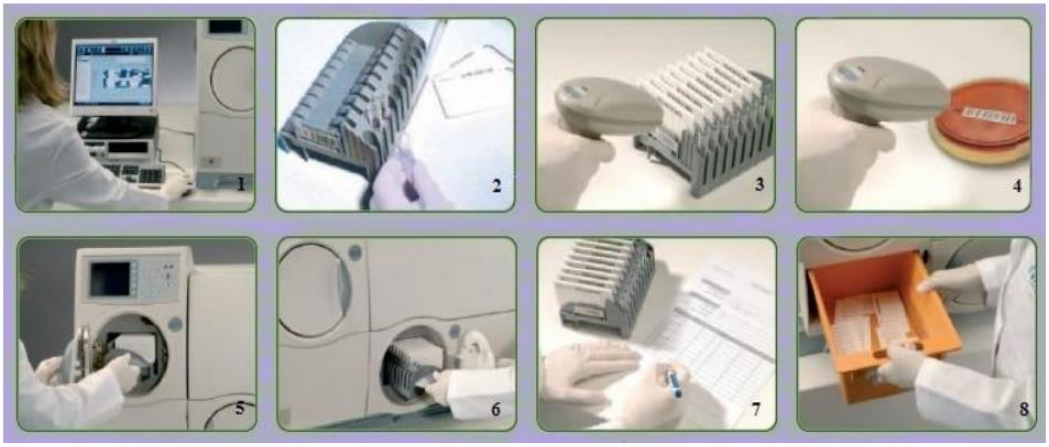

*Figure 4 - Detail of the VITEK®2.*

The VITEK®2 it's composed by [1] Computer hardware support; [2] Inoculation of the microbial suspension in the tubes and positioning of the identification letters in the cassette; [3] Identification of the card, by reading bar code; [4] Identification of the sample, which will be associated with a particular letter identified in the previous point; [5] The suspension is inoculated into the wells of the respective identification letter; [6] The cassette is transported to the player; [7] The results are automatically printed when the chart reading is complete and [8] After identification of the microorganism present in the bacterial suspension, the charts are removed from the equipment.

### 3.1.1. Identification of bacterial strains (ID)

The identification is made from a fresh and pure culture, from where a suspension of 0.55 to 0.65 McFarland of bacteria is achieved and inoculated through a vacuum system into the chosen cards. The cards are incubated at 35.5 ± 1 ° C and read every 15 minutes.

For identification, the results of a set of 64 tests are taken into account. To identify the microorganisms, there are four distinct cards available: 1) Gram-negative bacilli, fermenters or non-fermenters; 2) Gram-positive cocci and ex-non-sporulated bacilli; 3) yeasts; 4) cocci formed by Gram-positive spores. Each card has several wells containing biochemicals (evaluating the metabolic activity of the organism: acidification, alkalization, enzymatic hydrolysis or growth in the presence of inhibitors) and a well containing a growth control agent. The results are compared with a database of well-characterized strains, and an identification is obtained with a certain degree of similarity of the metabolic test.

The identification usually requires from 18h to 24h of incubation in the VITEK®2 Reader/Incubator.

### 3.1.2. Antimicrobial Susceptibility Testing (AST)

The antimicrobial susceptibility testing (AST) is indicated for all microorganisms that contribute to an infectious process and justify antimicrobial therapy. This test is based on the determination of minimal inhibitory concentrations (MICs) using different concentrations of antibiotics and is therefore determined from the lowest concentration at which inhibition of bacterial growth occurs. The AST was performed with the aid of VITEK®2 equipment.

The inoculum performed for the identification of the microorganism is sufficient to the antibiogram, and the suspension should have a density equivalent to a standard between 0.5 and 0.65 on the MacFarland scale for Gram-negative and Gram-positive bacteria. In all cards there is a control well, which contains only microbiological culture medium.

Each AST card has 64 microwells with selected antibiotics and in varying concentrations. The device monitors growth in each of the cards wells over a defined period of time (up to 18 hours for bacteria). The system determines which well shows growth of the microorganism based on the decrease in light intensity that is measured by the optical reader. At the end of the incubation cycle, an interpretive result (Sensitive, Intermediate or Resistant) relative to the antimicrobial agent will be marked together with an MIC, according to interpretations defined by the EUCAST guidelines.

### 3.1.3. Detection of extended-spectrum β-lactamases and AmpC β-lactamases producers microorganisms

*Escherichia coli* and *Klebsiella pneumoniae* species were screened for the presence of ESBLs. ESBL producers are identified by the automated system (VITEK®2) and were confirmed using phenotypic tests (E-test).

The suspected AmpC producers were selected based on the resistance to amoxicillin/ clavulanic acid.

## 4. Phenotipic methods

### 4.1. Detection of carbapenemases - RAPIDEC® CARBA NP

RAPIDEC® CARBA NP (BioMèrieux, France) is a rapid test that provides reliable results in less than 2 hours for carbapenemases producing bacteria. This has a high clinical value since it confirms the presence of bacteria producing carbapenemases. This allows to confirm the diagnosis and to help guide the treatment of the patient.

The RAPIDEC® CARBA NP test detects the hydrolysis of carbapenems by carbapenemase-producing bacteria: Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The hydrolysis acidifies the medium, which gives rise to the colour change of the pH indicator - specifically indicating the presence of resistance to transmissible carbapenems. It detects (without distinction) the 3 types of carbapenemases produced: KPC, metallo-β-lactamases (NDM-1, VIM, IMP) and carbapenemases of type OXA (Poirel and Nordmann 2015).

### 4.2. Ceftalozane/tazobactam

Minimal inhibitory concentrations (MICs) for Ceftolozane/Tazobactam were determined using a quantitative method – MIC Test Strip (MTS), with a predefined gradient of concentration that ranges between 0.016/4 - 256/4 μg/ mL, according to Liofilchem (Liofilchem, Italy) standards. MTS consists of special paper impregnated with a pre-defined concentration gradient of an antimicrobial agent, which is used to determine MIC in μg/ mL of antimicrobial agents against bacteria as tested on agar media using standard incubation temperature (35 ± 2 °C) and time (16–20 h).

The stored strains were plated with inoculation loop in PVX medium and incubated at 37 °C. A fresh and pure colony was taken, with a sterile swab, into the saline solution to achieve a suspension of 0,50 McFarland (for verification this density a densitometer was used). The suspension was than spread into a Mueller Hinton Agar plate, in all directions, with a sterile swab.

The excess of moisture was absorbed so that the surface was completely dry before applying MIC Test Strip. The strip was applied to the agar surface with the scale facing upwards and code of the strip to the outside of the plate, pressing it with sterile forceps on the surface of

the agar and ensure that whole length of the antibiotic gradient is in complete contact with the agar surface. Once applied, the strip should not be moved.

## 5. Genotyping methods

### 5.1. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was used to: verify the presence of *bla*$_{KPC}$, typing assays to investigate the clonal diversity of the isolates and screening for IncFIA plasmids, pBK30661 and pBK30683.

A DNA suspension was prepared by suspending one or two fresh colonies in 50 µL of sterile distilled water. A volume of 1 µL of suspension was added to a final volume of 24 µL PCR mixture.

Generally, PCR reactions were performed in 24 µL reaction mixtures containing 6.25 µL of NZYtech 2x Green Master Mix (NZYtech, Portugal), sterile distilled water, reverse primer and forward primer. The Master Mix contains deoxyribonucleotides phosphates (dNTPs), reaction buffer, $MgCl_2$ and Taq DNA polymerase. The Master Mix have 2.5 mM $MgCl_2$, 200 µM dNTPs and 0.2 U/ µl DNA polymerase. The primers used for each assay are shown in Table 1 - Primer sequences for PCRs in this study

A PCR scheme with four duplex PCRs was designed to detect pBK30661-like and pBK30683-like plasmids. Duplex I (PCR-1 and PCR-2) was designed to target the IncFIA plasmid-specific replication gene repA (in both pBK30661 and pBK30683) and the second IncFII replication gene repA in pBK30683. Duplex II (PCR-3 and PCR-4) was designed to target the upstream and downstream junctions between Tn*4401* and the neigh-boring regions of the plasmid. Duplex III (PCR-5 and PCR-6) was designed to detect both junctions of the 68-kb integrated fragment in pBK30683. Duplex IV (PCR-7 and PCR-8) was used to detect the pBK30661- and pBK30683-associated Tn*4401d* isoform. pBK30661-like plasmids are positive with PCR-1, PCR-3, PCR-4, PCR-6, PCR-7, and PCR-8, while pBK30683-like plasmids are positive with all eight PCRs (Figure 5) (Table 1) (Chen et al. 2014).

In each PCR experiment was used a negative control that differ from the reaction mixture by substituting the cell suspension by the same volume of $dH_2O$.

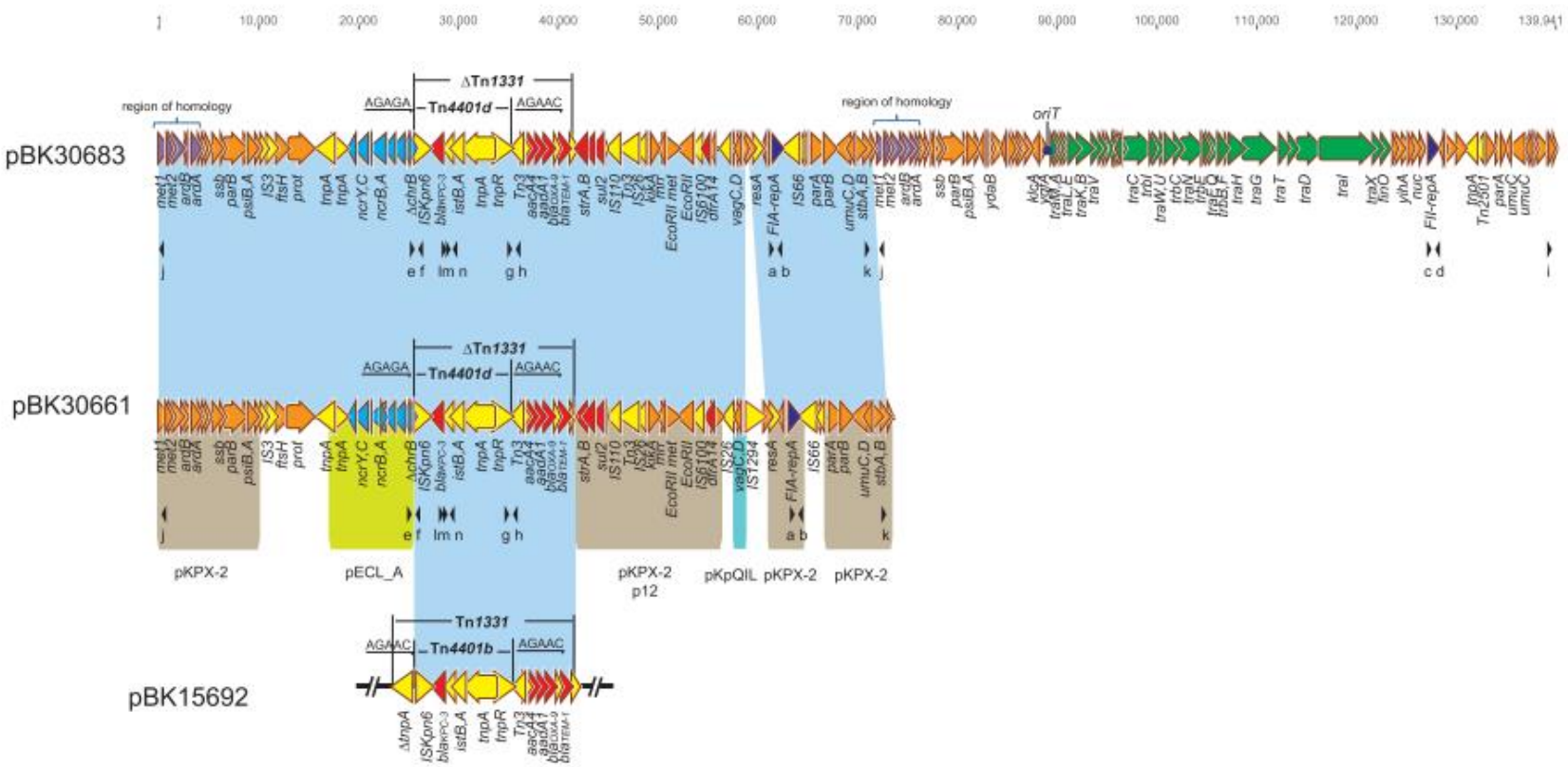


*Figure 5 - Plasmid structures of pBK30661, pBK30683 and the Tn4401/Tn1331 nested transposon in pBK15692. Light blue shading, shared regions of homology among pBK30661, pBK30683, and pBK15692 (Chen et al. 2014)*

*Table 1 - Primer sequences for PCRs in this study*

| | | Primer pair | Target | Primer sequence (5' - 3') | Amplicon size (bp) | Reference |
|---|---|---|---|---|---|---|
| | KPC | KPC_for<br>KPC_rev | KPC-1 to KPC-5 | CATTCAAGGGCTTTCTTGCTGC<br>ACGACGGCATAGTCATTTGC | 538 | (Bouchillon et al. 2013) |
| | BOX-PCR | BOX A1R | generate BOX-PCR profiles | CTACGGCAAGGCGACGCTGACG | | (Weisburg et al. 1991) |
| IncFIA | PCR-1 | IA-1f (a)<br>IA-1r (b) | IncFIA repA of pBK30661 and pBK30683 | GCCGTCCTTTCTGTGACAAATCA<br>GGATGGACTGTGGGCACGTT | 516 | (Figure 5)<br>(Chen et al. 2014) |
| | PCR-2 | IA-2f (c)<br>IA-2r (d) | Second IncFII repA in pBK30683 | CCGTTTCTGTGTCATTTGCTCCT<br>CTTATAGTGAGACGGCCGGAACC | 250 | |
| | PCR-3 | IA-3f (e)<br>IA-3r (f) | Tn*4401* upstream junction between *chr*B gene and IS*Kpn6* in both pBK30661 and pBK30683 | ATACCGGTGCCGCCATGCTGCG<br>TCGTCATGCCGCGGACCACCCC | 213 | |
| | PCR-4 | IA-4f (g)<br>IA-4r (h) | Tn*4401* downstream junction between Tn*4401 tnpR* gene and neighboring Tn*3 tnpA* gene in both pBK30661 and pBK30683 | CCGGCATCACCGGCCCTCACCT<br>ACACTCCCGGCTGTGCGCCTGA | 515 | |
| | PCR-5 | IA-5f (i)<br>IA-56r (j) | Region between putative cytoplasmic protein gene and adenine-specific methyltransferase gene (met1) in pBK30683 | CGATGACGTGGAGAGCAGTA<br>TCCCGAGAATGAATCTGGAC | 534 | |
| | PCR-6 | IA-6f (k) | Region between hypothetical protein gene and adenine- specific methyltransferase gene (met1) in both pBK30661 and pBK30683 | CGTGCATTCGGTGACTAAAA | 768 | |
| | PCR-7 | 4401v-r (3781L) (l)<br>4401v-r1 (m) | Tn*4401d* isoform in pBK30661 and pBK30683 | CACAGCGGCAGCAAGAAAGC<br>GCAAGCCGCTCCCTCTCCAG | 635 | |
| | PCR-8 | 4401v-f (3098U) (n) | | TGACCCTGAGCGGCGAAAGC | 314 | |

PCR was performed in a MyCycler Thermal Cycler (Bio-Rad, USA), with different temperature and time for all steps (Table 2). A DNA molecular weight marker, GeneRuler™ DNA Ladder Mix - Thermo Fisher Scientific was used as DNA size standard. All primers used were ordered from Stab Vida, Portugal or from Sigma, USA.

*Table 2 - PCR programs used for amplifications.*

| | KPC gene | | Strain typing | | IncFIA plasmid | | |
|---|---|---|---|---|---|---|---|
| Initial denaturation | 94℃ | 5 min | 94℃ | 7 min | 95℃ | 4 min | |
| Annealing | 94℃ | 30 seg | 94℃ | 1 min | 95℃ | 30 seg | x30 |
| | | | 53℃ | 1 min | 60℃ | 30 seg | |
| | 55℃ | 30 seg | 65℃ | 8 min | 72℃ | 1 min | |
| Final extension | 72℃ | 1 min | 65℃ | 16 min | 72℃ | 7 min | |

The resulting PCR products along with the DNA ladder were loaded in wells of 1.5% agarose gel prepared in 1X Tris EDTA (TAE) buffer. Gel electrophoresis was used to separate DNA fragments, 90 V for 1 hour for detection *bla*$_{KPC}$ and IncFIA plasmid, and 90 V for 1 h30 for genotyping. The gel was then stained with ethidium bromide for 15 minutes and in $dH_2O$ for 10 min and visualized under UV light. Molecular Imager® Gel Doc™ XR (Bio-Rad, USA) was used to scan the gel.

# Chapter I.

# Epidemiology of carbapenemases-producing bacteria in Centro Hospitalar do Baixo Vouga

**Abstract:** Infectious diseases caused by bacteria are one of the major health problems. Moreover, the emergence of β-lactamases, namely, carbapenemases, such as KPC, that confer resistance to most β-lactam antibiotics are motive of concern. The aim of this study was to evaluate the presence of *bla*$_{KPC}$ in strains collected in Centro Hospitalar do Baixo Vouga, E.P.E., Portugal, from inpatients and outpatients.

All the isolates included in this study are non-duplicate and were identified using Gram-negative bacteria identification card of the automated system VITEK®2 (BioMèrieux, France), antimicrobial susceptibility testing (AST) was estimated and advanced expert system (AES) rules were taken in consideration. Strains carbapenem resistant were studied by PCR to investigate the presence of the *bla*$_{KPC}$ and typing bacteria. Strains with a positive result in *bla*$_{KPC}$ were further studied to investigate *bla*$_{mcr-1}$ and IncFIA plasmid. RAPIDEC® CARBA NP (BioMèrieux, France) test was used, according to manufacture's instructions, to confirm the presence of a carbapenemase.

During the timeframe of this study (2017-2019) a total of 6189 strains were isolated among them 61 Enterobacteriaceae and three *Pseudomonas aeruginosa* exhibited resistance to the carbapenems. Fifty-nine strains were recovered from outpatients and five were recovered from inpatients. Patients age ranged from 43 to 94 years old. Among all the isolates, *Klebsiella pneumoniae* was the most relevant species since 52 strains were identified. The remaining isolates were: five *Enterobacter cloacae*, one *Escherichia coli*, one *Citrobacter freundii* and one *Enterobacter aerogenes*. The PCR was positive for the presence of the *bla*$_{KPC}$ in 50 of 52 strains, which were all *Klebsiella pneumoniae*. The RAPIDEC® CARBA NP test was positive.

The strains of *K. pneumoniae* were divided into 26 clusters, *E. cloacae* into four different clusters and *P. aeruginosa* into two clusters by BOX-PCR.

A PCR scheme to detect the distribution of *bla*$_{KPC}$-harboring IncFIA (pBK30661-like and pBK30683-like) plasmids in the collection of clinical isolates when they present the *bla*$_{KPC}$ was followed. KPC-harboring IncFIA plasmids were found in 27 of 52 *K. pneumoniae* isolates.

The results of this study show that in our region, as expected, the *bla*$_{KPC}$ was prevalent in the *K. pneumoniae* and the majority was transferred in IncFIA plasmid. Nonetheless, the percentage of carbapenems resistant Enterobacteriaceae remains low (1 %). Moreover, it is extremely important to apply earlier diagnostic techniques, such as molecular techniques, that will help to contain the spread more rapidly.

**Key-words:** carbapenems, carbapenemases, *bla*$_{KPC}$, Enterobacteriaceae, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, IncFIA plasmid

## 1. Introduction

Antibiotics are necessary for overall practice of medicine reducing morbidity and mortality (Van Hoek et al. 2011; Khan et al. 2018). Nowadays, the number of different antimicrobial agents are known is large (Van Hoek et al. 2011). However, β-lactams remain the major antibiotic class applied in the treatment of infectious diseases (Moya et al. 2009).

Carbapenems are widely used because they are a broad spectrum of antibacterial activity (Bina et al. 2015). In addition, carbapenems were recommended for the treatment of infections caused by multi-drug resistant (MDR) microorganisms (Mohamed et al. 2018).

However, in the preceding decade, there was an increase in the antimicrobial resistance in different pathogens. Microorganisms have several mechanisms that allow them to adapt to stress and lead to resistance to several antimicrobials used in clinical practice, for example, strains resistant to carbapenems have been isolated (Khan et al. 2018; Mohamed et al. 2018).

Resistance to carbapenems in Gram-negative bacteria results essentially from a decrease in bacterial outer membrane permeability, increased in extended-spectrum β-lactamases (ESBLs), AmpC β-lactamases production and carbapenemases (Bina et al. 2015).

Carbapenemases are enzymes that hydrolyse carbapenems and are able to break penicillins, cephalosporins and monobactams (Logan 2012). In Enterobacteriaceae, these enzymes are the most important mechanism of carbapenems resistance. In addition, these are high capacity to disseminate, which leads to a high concern in the prevention and transmission of these microorganisms (Bina et al. 2015; Bonomo et al. 2018).

Carbapenemases like KPC, NDM and OXA demand special attention (Bina et al. 2015). For example, KPC is the most common enzyme disseminated among Enterobacteriaceae, since often are carried on a mobile plasmid. Also, plasmid that mediates NDM has spread quickly globally and into that family (Logan 2012). So, carbapenem-resistant Enterobacteriaceae (CRE) have become widespread globally and are an urgent public health threat (Khan et al. 2018).

Moreover, *bla*$_{\text{KPC}}$ are commonly located on Tn*4401*. *bla*$_{\text{KPC-3}}$ was part of Tn4401 isoform d and located in a cointegrated FIA and FII plasmid. In Portugal, the FIA plasmid was reported in clinical isolates, like *K. pneumoniae, E. coli,* and *Enterobacter* spp. (Rodrigues et al. 2016; Tacão et al. 2017).

## 2. Results and discussion

### 2.1. Characterization of bacterial strains

In this study, 64 strains resistant to carbapenems were collected, recovered during 2017 (N=34), 2018 (N=25), and until March 2019 (N=5).

Overall samples, the number of strains collected from women (n=37) is higher than number of isolates from men (n=27) (Figure 6).

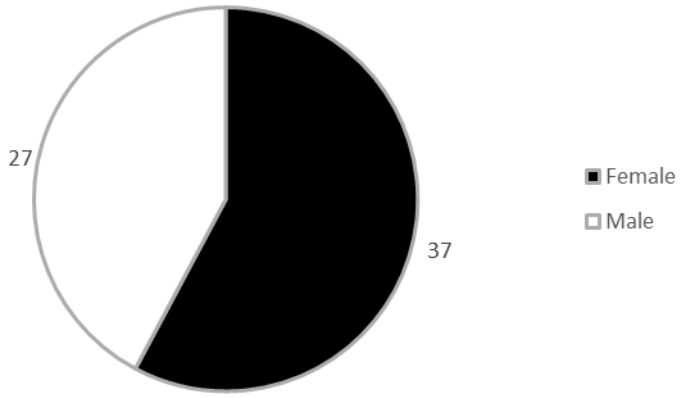


*Figure 6 - Isolates resistant to carbapenems distributed by gender*

The patient ages ranged between 43 and 94 years old, being the average age of patients with carbapenem- resistant microorganisms 75 years old. These results demonstrate that the population with carbapenemase-producing organisms (CPOs) tends to be older and are according to Duin and Doi 2017, in the Consortium on Resistance Against Carbapenems in *Klebsiella* and other Enterobacteriaceae (CRACKLE), the average age of patients in the same conditions was 70 years old (age between 58 to 81 years).

All the isolates were non-duplicate and recovered from colonization or infections sites: urine (n=46), pus (n=9), blood (n=6), sputum (n=2) and tip of the catheter (n=1).

Following the criteria described in Material and Methods section, 2, the microorganisms were classified as nosocomial or non-nosocomial. Overall 64 isolates collected, five were nosocomial in different ward of CHBV, whereas 59 were non-nosocomial (Figure 7).

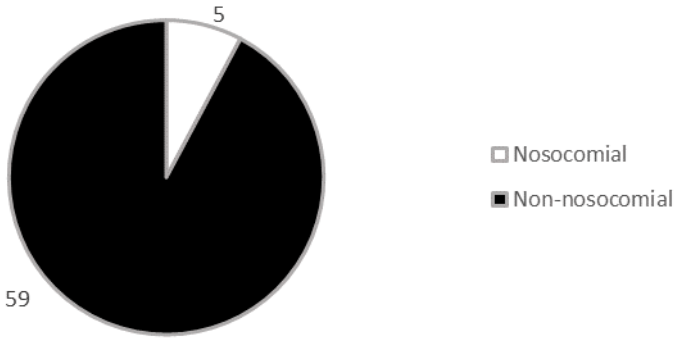


*Figure 7 - Distribution of the carbapenemase-producing bacteria considering if they were nosocomial or non-nosocomial*

The data of this study strengthens an ongoing dissemination of CPOs in community. This fact may suggest that cross acquisition and/or a common exogenous source is an important route of CPOs acquisition.

During the period of this study, a total of 64 microorganisms, including 52 *K. pneumoniae*, six *E. cloacae*, three *P. aeruginosa* and one *E. coli*, *E. aerogenes* and *C. freundii* were suspected to have the *bla*$_{KPC}$ since they are resistant to carbapenems.

## 2.2. Detection of *bla*$_{KPC}$

### 2.2.1. *Klebsiella pneumoniae*

Among 52 *K. pneumoniae* analysed, the *bla*$_{KPC}$ was present in 50 of isolates (Figure 8).

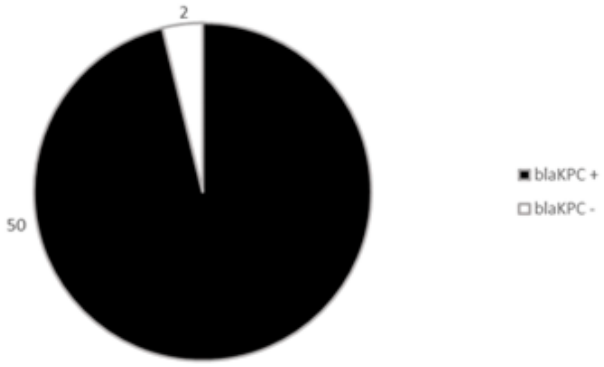


*Figure 8 - Presence (bla$_{KPC}$ +) or absence (bla$_{KPC}$ -) in K. pneumoniae isolates*

This fact can justify that resistance to carbapenems, since it is linked to different mechanisms, including the production of enzymes like KPC, VIM, IMP, NDM, and OXA- 48 and among others (Weinstein and Logan 2012; Girmenia et al. 2016).

In this study the primers used cover from *bla*$_{KPC-1}$ to *bla*$_{KPC-5}$ and is describe that genes *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$ account for most of *bla*$_{KPC}$. Nonetheless, it not possible to describe the positive results in such detail. However, in Portugal, *bla*$_{KPC-2}$ was identified only in an environmental *E. coli* isolate in 2010, while *bla*$_{KPC-3}$ producers were first detected in 2009 in a central hospital. Recently, have been verified widespread distribution of *bla*$_{KPC-3}$ among *K. pneumoniae* isolates in different Portuguese hospitals (Rodrigues et al. 2016; Duin and Doi 2017).

#### 2.2.1.1. Susceptible profile

Among the susceptibility profile for each isolate, special attention was give to last resource antibiotics. Taking that in to account the following results were retrieved: the highest and lowest resistance was related to piperacillin/tazobactam (n=52/52) and meropenem (n=51/52); and colistin (n=5/52), respectively. Intermediate resistance to gentamicin, piperacillin/tazobactam and colistin was not observed (n=0/52) and intermediate resistance was very low (1.6%) for meropenem (n=1/52), ceftazidime (n=4/52) and cefepime (n=7/52).

Colistin was the antibiotic with the highest number of susceptible isolates among *K. pneumonia* (n=47/52), on the other hand meropenem, ceftazidime, cefepime and piperacillin/tazobactam, exhibited the lowest sensitivity (Table 3).

*Table 3 - Antibiotic resistant by K. pneumoniae isolates. MER: meropenem; CFT: ceftazidime; CP: cefepime; GM: gentamicine; PIP/TAZ: piperacillin/tazobactam; COL: colistin*

| Result | Antibiotic resistant (n/ n total) | | | | | |
|---|---|---|---|---|---|---|
| | MER | CFT | CP | GM | PIP/TAZ | COL |
| Resistance | 51/52 | 48/52 | 45/52 | 36/52 | 52/52 | 5/52 |
| Intermediate | 1/52 | 4/52 | 7/52 | 0/52 | 0/52 | 0/52 |
| Sensitive | 0/52 | 0/52 | 0/52 | 16/52 | 0/52 | 47/52 |

Additionally, the presence of *bla*$_{\text{mcr-1}}$, *bla*$_{\text{mcr-3}}$, *bla*$_{\text{mcr-4}}$ and *bla*$_{\text{mcr-5}}$ was accessed by PCR method. Among the five isolates that were resistant to colistin (Kp1, Kp11, Kp37, Kp47 and Kp44), the gene mcr-1 was shown to be present in one strain (Kp11).

Also, plasmid IncX-4 was screened in the isolate, but this wasn't identified (Data not show).

### 2.2.2. Other Enterobacteriaceae

Among all isolates of *E. cloacae*, *E. aerogenes*, *E. coli* and *C. freundii* the *bla*$_{\text{KPC}}$ was not found. Other studies report that for *Enterobacter* spp., resistance to carbapenems was associated with β-lactamase expression (VIM producers are most often found in Italy and Greece) and porin deficiency in combination, with a variety of deletion, point mutation, insertional inactivation and unknown mechanisms leading to the loss of porin expression (Doumith et al. 2009; Bonomo et al. 2018).

This can be justified since have already been reported in Enterobacteriaceae a class D carbapenem-hydrolyzing β-lactamases, namely, *bla*$_{\text{OXA-48}}$. Nowadays, are acknowledged and are increasingly reported worldwide, in many Gram-negative bacteria, including Enterobacteriaceae, notably, community *E. coli* isolates (Djahmi et al. 2014; Bonomo et al. 2018). In addition, OXA-48-like carbapenemases remain extremely rare as a cause of carbapenem resistance in Enterobacteriaceae in the US. However, they are relatively commonly found in Europe, especially in Mediterranean countries (Duin and Doi 2017).

### 2.2.3. *Pseudomonas aeruginosa*

*bla*$_{\text{KPC}}$ was not found in the *P. aeruginosa* (n=3) isolates as it was expected, since metallo-β-lactamases (MBLs) are commonly identified in *P. aeruginosa*. However, in the absence of carbapenemases, the resistance to carbapenems is usually multifactorial: increased production of AmpC chromosome-encoded cephalosporinase, reduced outer membrane porin OprD expression and associated factors are known to contribute to carbapenem resistance (Poirel and Nordmann 2009).

### 2.3. Strain typing: BOX analysis

The clonal composition of 64 isolates was evaluated by BOX-PCR. Among 52 strains of *K. pneumoniae* examined it was possible to identify 26 different banding patterns (1-11 isolates each, shown in Figure 9); five different banding types of *E. cloacae* isolates (1–2 isolates each) and two different banding types of *P. aeruginosa* isolates (1-2 isolates each).

Sixteen *K. pneumoniae* isolates exhibited unique genotypes, that confirm the discriminatory power of the BOX-PCR technique. This is revealed by Figure 9, which suggests a considerable high heterogeneity and genomic diversity between the clinical isolates.

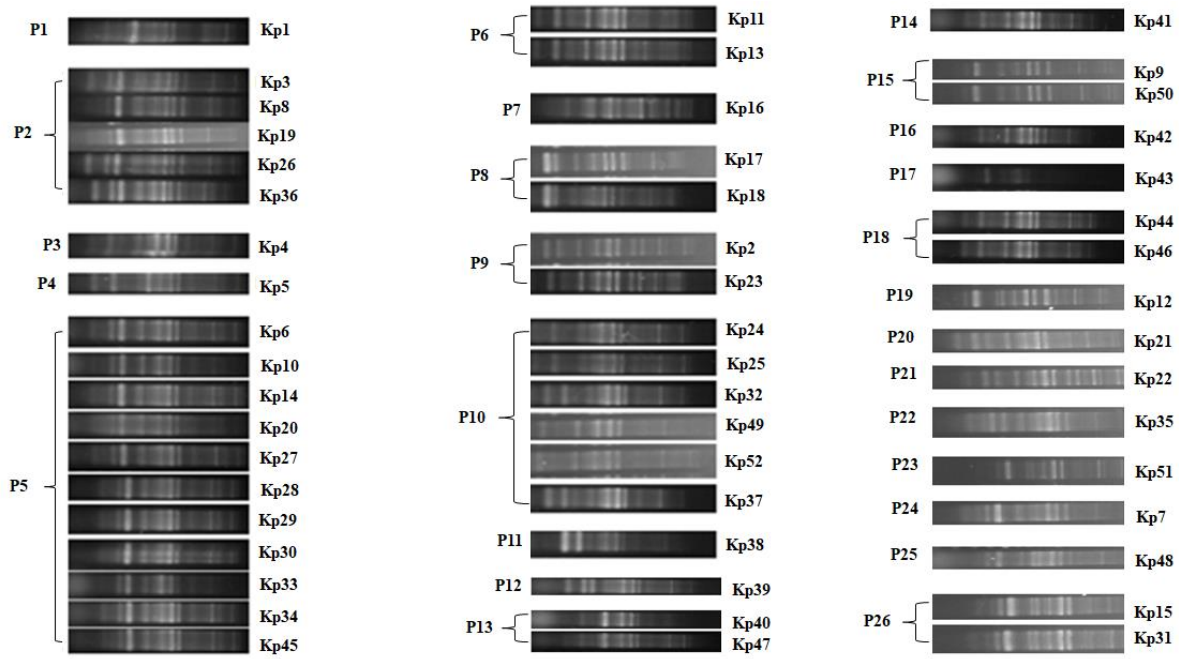


*Figure 9 - Isolates of K. pneumoniae grouped by typing, performed by BOX-PCR*

### 2.4. Detection of plasmid IncFIA

Strains representative of different clusters and positive for the presence of *bla*$_{KPC}$ were screened for IncFIA plasmids, pBK30661 and pBK30683.

When one of the plasmids was identified, was considered present in all strains belonging the same cluster; the same happens when plasmids were not found. Among 52 isolates of *K. pneumoniae*, the pBK30683 plasmid was found in 27 strains included in 11 different clusters.

The presence of pBK30683 indicates that *bla*$_{KPC}$ is located on Tn*4401d*. These results will confirm the role of Tn*4401d* on the spread of carbapenemase genes among in Portugal, as previously verified (Tacão et al. 2017). Acquisition of *bla*$_{KPC-3}$ by plasmids from different incompatibility groups, like IncFIA, appear to be epidemic and favoured a quick intra- and inter-species dissemination (Chen et al. 2014; Rodrigues et al. 2016).

## 3. Conclusion

The recent emergence of CRE is a major threat to hospitalized patients and continues to cause serious multidrug-resistant infections around the world (Sartelli et al. 2014). CRE and *P. aeruginosa* have recently increased in some European countries and they frequently harbour resistance mediated by carbapenemase production (Sader et al. 2014).

Carbapenemase genes usually are associated with mobile elements that encode resistance to several antibacterial drugs, and consequently produce multi-resistance in strains and dissemination interspecies and intraspecies is facilitated. This scenario might predict the emergence of drug-resistant phenotypes, likely jeopardizing treatment (Chen et al. 2014; Sader et al. 2014; Tacão et al. 2017).

In this study was verified that *bla*$_{KPC}$ is the most prevalent among strains.

Moreover, further studies are required to determine the origins of the pBK30661 plasmid and the distributions of this plasmid group in other geographic areas, in order to understand their contributions to KPC epidemiology.

In conclusion, is necessary to reinforce of infection control measures, surveillance, and tracking of isolates resistant to carbapenems in clinical institutions, and a coordinated action between clinicians, epidemiologists and national reference laboratories for guidance and harmonization of protocols.

# Chapter II.

# Dissemination of AmpCs in Isolates Causing Urinary Tract Infections and Skin and Soft Tissues Infections in Centro Hospitalar do Baixo Vouga

**Abstract:**

Urinary tract infections (UTIs) and skin and soft tissues infections (SSTIs) are associated with considerable levels of morbidity, mortality and leading to an increase in healthcare expenditure. In the past two decades has been witnessed an increasing prevalence of resistant pathogens in healthcare settings that cause these infections.

One of the most important mechanisms of resistance is AmpC β-lactamases. These cephalosporinases are clinically important, since mediate resistance to commonly used antimicrobial agents, like, penicillins and their derivates. They are worrisome since can be chromosomally encoded and found in plasmids, leading to their easy dissemination around the world. Furthermore, sometimes, in non-nosocomial infections they render the antimicrobial empiric therapy improper.

The aim of the present study was to evaluate the dissemination of AmpC β-lactamases in strains from urinary tract and soft tissues infections. Also, it was an objective to infer on the origin of these infections.

A total of 155 strains AmpC-producing Enterobacteriaceae were isolated from patients with UTIs and SSTIs in Centro Hospitalar do Baixo Vouga, since September to December 2018. Strains resistant to amoxicillin/ clavulanic acid (detected by automated test VITEK®2) were selected as it is indicative the presence of $bla_{\text{AmpC}}$.

In this study, has been verified *Klebsiella pneumoniae* and *Escherichia coli* were the most common Gram-negative pathogens isolated from UTIs and SSTIs. Others Enterobacteriaceae were also present in these infections, such as *Enterobacter cloacae*, *Morganella morganii*, *Klebsiella oxytoca*, among others. In addition, most of these infections are of non-nosocomial origin.

This problem leads to complications in choosing the appropriate treatment and it is of utmost importance.

**Key-words:** urinary tract infections, skin and soft tissues infections, AmpC β-lactamases, dissemination, cephalosporinases, Enterobacteriaceae, Gram-negative microorganisms, non-nosocomial

## 1. Introduction

Urinary tract infections are the most common infection both in the younger and older population. In 2010, the UTIs was the fourth foremost reason for hospital admission in older patients, followed heart failure, pneumonia and septicemia (Ma and Hughes 2018). There are several diseases associated with a urinary tract that encompasses infections from simple cystitis involving the bladder to the entire urinary tract, including renal pelvis and kidney (pyelonephritis) (Smirnov and Egbert 2016).

Skin and soft tissues infections (SSTIs) are amongst the most common bacterial infections in humans and represent about 10% of hospital admissions in the US. SSTIs has a broad range of etiology, clinical manifestation and severity (Eckmann and Dryden 2010). The skin and soft tissues infection in hospitalized patients contributes to prolonged length of stay, leading to increase costs of medical care and antimicrobial resistance emergence (Moet et al. 2007). Both, the skin and soft tissue is a resistant, flexible and structural barrier to invasion. Ruptures in skin, like ulcers and surgical or traumatic wounds, allow colonization with a broader range of bacteria, which can progress in inflammation of surrounding areas - soft tissues. In other words, skin and soft tissue infections are related microbial invasions in epidermis, dermis and subcutaneous tissues and can be potentially fatal (Dryden 2009, 2010).

Most prevalent microorganisms in UTIs are *E. coli* (75%-95% of events), followed by *Pseudomonas aeruginosa*, *Klebsiella* spp., Enterobacter species and Acinetobacter *baumannii*. *E. coli* strains infect urinary tract through a variety of mechanisms, including special adhesions, fimbriae, biofilm and aversion of host responses (Peleg and Hooper 2012; Ma and Hughes 2018).

On the other hand, almost all microorganisms are able to cause SSTIs, but the major microorganism involved is *Staphylococcus aureus*, followed by *P. aeruginosa*, *E. coli* and *Enterococcus* spp. (Dryden 2009).

For optimal treatment of these infections is necessarily know pathophysiology, awareness of microorganisms that cause infection, find adequate antibiotic therapy and if necessaire reaches for surgical intervention (Dryden 2010; Ma and Hughes 2018).

As mentioned above, Gram-negative microorganisms are associated of UTIs and SSTIs; and are highly efficient in the acquisition of drug resistance mechanisms, namely, β-lactamases that destroy the β-lactam ring (Llarrull et al. 2010; Peleg and Hooper 2012). In addition, has been reported an increase in the number of drug-resistant organisms that cause SSTIs in both hospital- and community-acquired (Eckmann and Dryden 2010).

AmpC β-lactamases are clinically important cephalosporinases since they mediate resistance to most penicillins, some cephalosporins, some narrow-spectrum β-lactams and β-lactam/ β-lactamase inhibitor combinations (Jacoby 2009; Harris 2015; Iredell et al. 2016). Initially, *bla*$_{\text{AmpC}}$ has been described only in chromosome, but nowadays *bla*$_{\text{AmpC}}$ is present in plasmids and has the ability to transfer between them and it dissemination is observed worldwide (Bush et al. 1995; Jacoby 2009; Maseda et al. 2014). So, microorganisms with potential for production AmpC β-lactamases may lead to multidrug resistance, which difficult the selection of an effective antibiotic and treatment (Jacoby 2009).

β-lactam/ β-lactamase inhibitors are used for empiric treatment of UTIs and SSTIs. However, some β-lactamases inhibitor enhance the expression of the AmpC, such as clavulanic acid, in other words, AmpC β-lactamases are inducible by clavulanic acid (Jacoby 2009). Empiric antimicrobial therapy was inappropriate in more than 30% of the cases of an infection caused by a pathogen identified to be an AmpC-producer (Conen et al. 2015).

Cefepime, ceftolozane and carbapenems are stable against AmpC β-lactamases therefore can be used to treat these type of infections (Jacoby 2009; Khan et al. 2018).

## 2. Results and discussion

### 2.1. Bacterial strains characterization of urinary tract infections

In this study, 133 Enterobacteriaceae with AmpC were collected from a positive uroculture.

Among all samples, the number of urine samples retrieved from women (n=76) is higher than number of isolates from men (n=57) (Figure 10), which is in agreement with the studies performed by Mendo et al. 2008 and Costa et al. 2009.

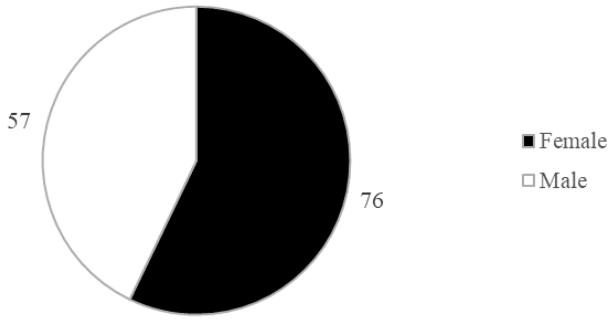


*Figure 10 - Urinary tract infection distributed by gender.*

This result can be explained by the structural or functional differences of the urinary tract between men and women, such as, length of the urethra and its close proximity to the vagina. Also due to sexual activity, gestation and menopause. On other hand, UTIs are less frequent in males given that they possess a longer urethra and due to the antibacterial action of prostatic liquid. When these infections occur they may be connected to complex problems such as prostate obstructions, vesicular calculus, catheterization and diabetes (Mendo et al. 2008).

The distribution of the strains collected by age shows that 68% are 65 years of age or older (90/133 isolated), 15% are between 19 and 64 years old (20/133) and 17% of the population are less than 30 years (23/133 isolated) (Figure 11). The youngest is one week old and eldest has 97 years old, demonstrating high heterogeneity in the group of individuals under study regarding age.

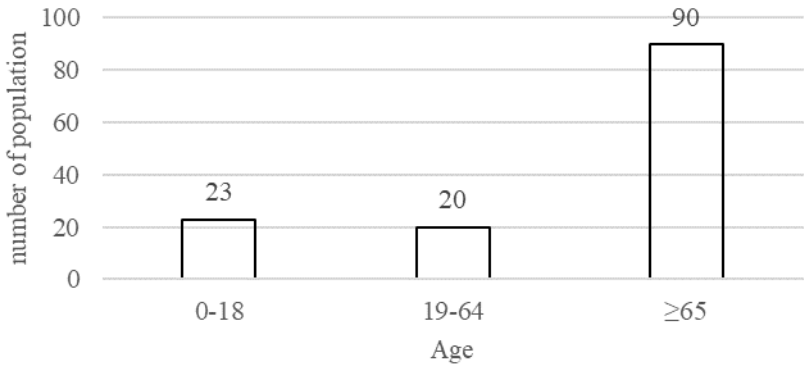


*Figure 11 - Distribution of the strains collected by age in urinary tract infections*

The incidence was higher in patients with 65 years of age or older. This can be explained, due to the increase in the immune systems weakness, possible limitations of hygiene habits and anatomical-functional alterations of the bladder between men and women, such as partial obstruction of the urinary tract, manipulation, reduction of urinary flow and anomalies (Mendo et al. 2008; Costa et al. 2009; Narciso et al. 2011).

Following the criteria described in Material and Methods section, 2, the microorganisms were classified as nosocomial or non-nosocomial. Overall 133 isolates collected, 42 were nosocomial in different ward of CHBV, whereas 91 were non-nosocomial (Figure 12).

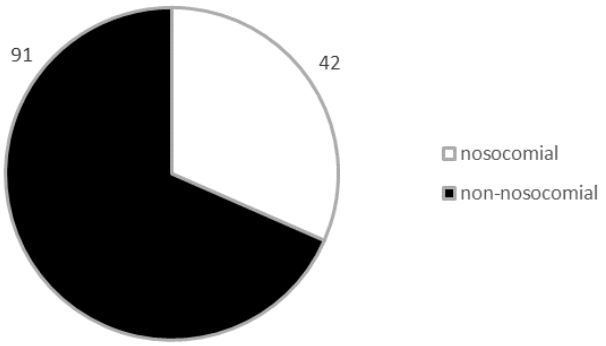


*Figure 12 - Distribution of the UTIs isolates considering if they were nosocomial or non-nosocomial*

The study reported by Bouchillon et al. 2013, describes the same trend, among 2,135 Gram-negative isolates, the number of hospital-acquired (HA) isolates is lower (20%) than community-acquired (CA) isolates (66%). These results can be explained on the basis of an erroneous empirical treatment that leads to their successful spread in the community (Conen et al. 2015).

Among all isolated species, *K. pneumoniae* (n=58) and *E. coli* (=57) were the most common pathogens isolated from urine, followed by *E. cloacae* (n=8), *Morganella morganii* (n=4), *K. oxytoca* (n=2) and *E. aerogenes* (n=2); and *Serratia marcescens* (n=1) and *C. freudii* (n=1), as shown in Figure 13.

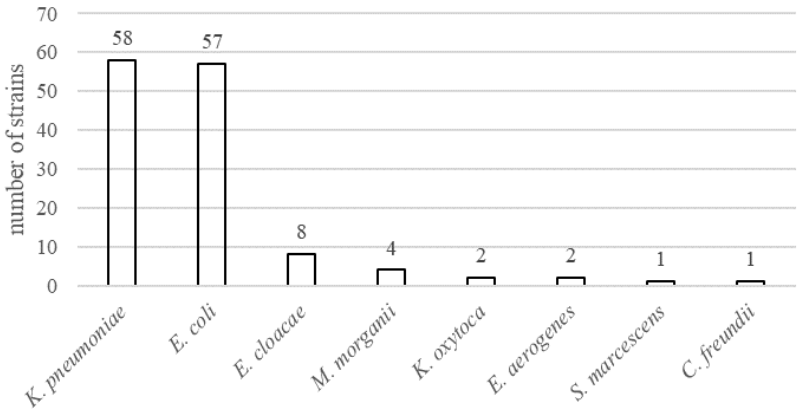


*Figure 13 - Distribution of different strains probably caused urinary tract infections, among all isolates tested positive for AmpC expression*

Uropathogenic identified in this study were similar to those of many other studies. According to other studies *E. coli* was the most frequently isolated pathogen causing UTIs, followed *K. pneumoniae*. As well as, other Enterobacteriaceae species, including *C. freundii*, *C. koseri*, *E. aerogenes*, *M. morganii* and *S. marcescens*, *P. mirabilis*, *P. aeruginosa*, *K. oxytoca* and *E. cloacae* (Bouchillon et al. 2013; Morrissey et al. 2013; Jean et al. 2016; Salleh et al. 2017).

In the present study, the carriage of an AmpCs alone (n=70), both an AmpCs and an ESBL (n=45) and both an AmpCs and CPOs (n=18) was analysed (Figure 14).

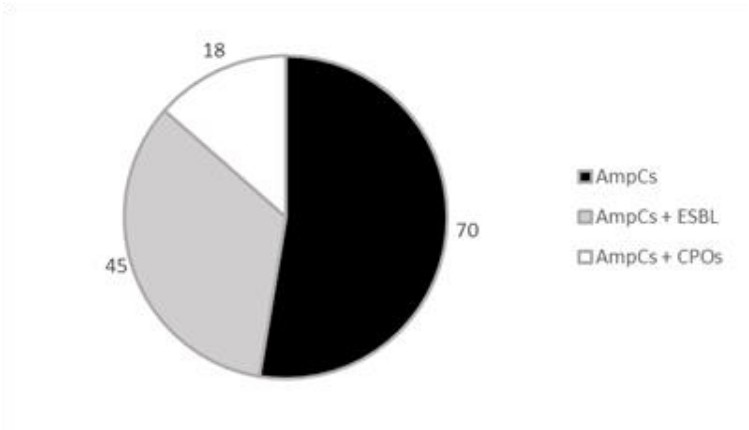


*Figure 14 - Distribution of combined resistance mechanisms among AmpCs positive strains*

Among 113 strains with AmpCs, 45 was confirmed to also produce extended-spectrum β-lactases (ESBLs), namely, *K. pneumoniae* (30/45), *E. coli* (14/45) and *K. oxytoca* (1/45) (Figure 15).

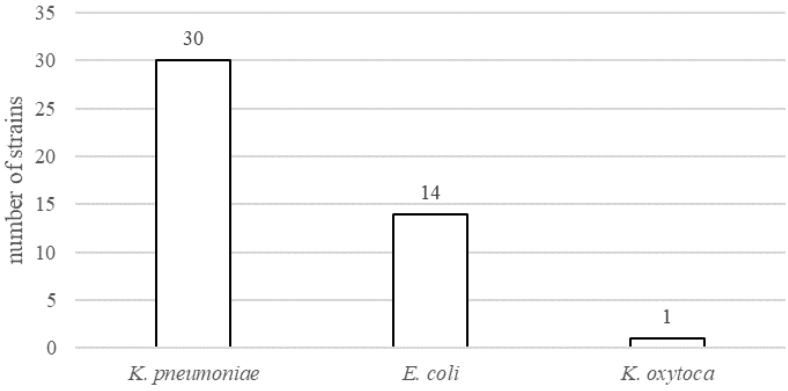


*Figure 15 - Distribution of strains producer both AmpC and ESBL.*

According to Jacoby 2009, most of the times organisms producing AmpC β-lactamases are also positive ESBLs. In addition, according to Bouchillon et al. 2013, ESBLs rates were 10.3% of *K. pneumoniae*, 6.8% of *E. coli*, 11.1% of *K. oxytoca* and 3.7% of *P. mirabilis* isolates. These strains are very important since ESBL-producing *K. pneumoniae* was associated with a higher rate of treatment failure 72h after the initiation of treatment when compared with non-ESBL (Kang et al. 2004).

Also, in this study we analysed the number of isolates carrying a combination of an AmpCs with CPOs. Among 113 Gram-negative strains collected with AmpCs, 17 *K. pneumoniae* and one *E. cloacae* were resistant to carbapenems.

### 2.2. Bacterial strains characterization of skin and soft tissues infections

In this study, were collected 42 Enterobacteriaceae with AmpC, form soft tissues infections.

Figure 16 shows the analysis of the distribution of the strains, that cause or probably caused SSTIs, collected by gender. It was observed the number of strains were isolated from sputum samples retrieved from men (n=23) is higher than a number of isolates from women (n=19).

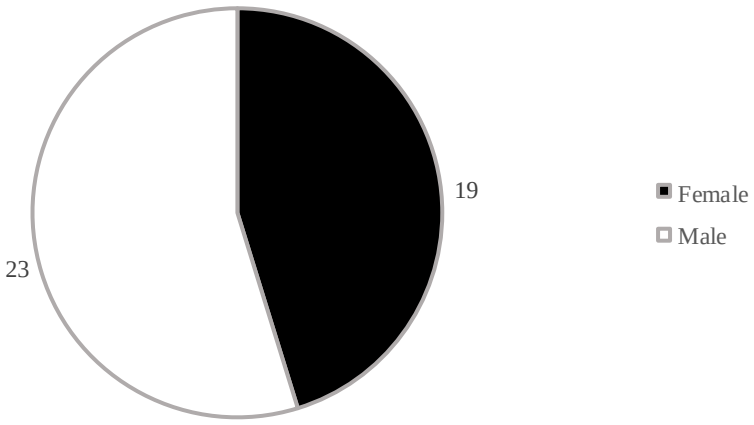


*Figure 16 - Skin and soft tissues infections distributed by gender.*

The distribution of the strains collected by age shows that 32/42 of isolated are 65 years of age or older, 8/42 strains are between 30 and 64 years old and 2/42 isolates of the population are less than 30 years (Figure 17). The youngest patient has two years and the eldest has 89 years old. Once again, these results demonstrate a high heterogeneity in the group of individuals under study regarding age. A study carried out by (Suaya et al. 2013) shows that SSTIs and their associated complications were more common in the population aged 65 years and older.

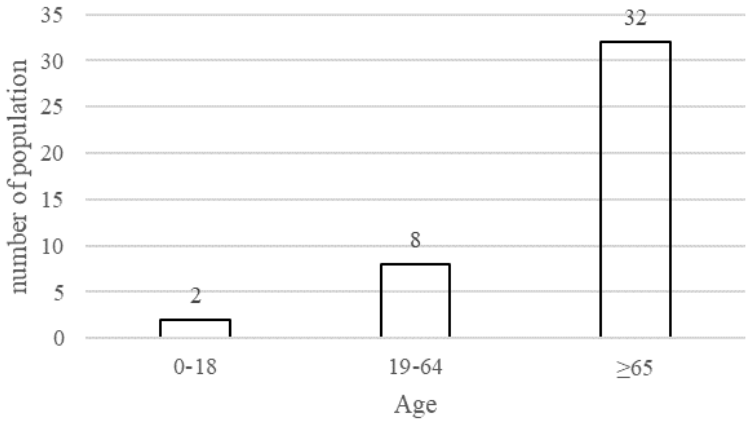


*Figure 17 - Distribution of the strains collected by age in soft tissues infections*

Following the criteria described in Material and Methods section, 2, the microorganisms were classified as nosocomial or non-nosocomial. Overall 42 isolates collected, 40 were non-nosocomial in different ward of CHBV, whereas two were nosocomial (Figure 18).

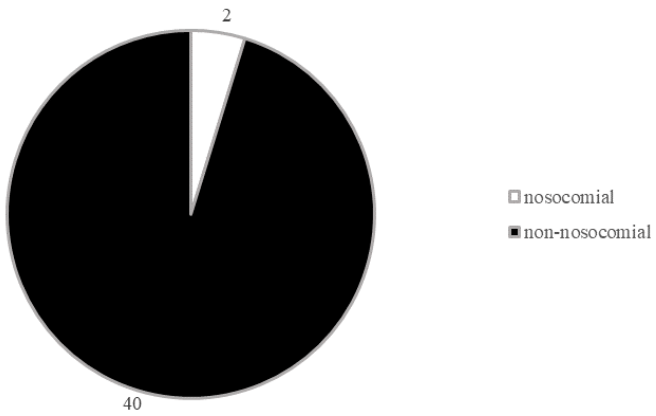


*Figure 18 - Distribution of the SSTIs isolates considering if they were nosocomial or non-nosocomial.*

Among all 42 isolated species, *K. pneumoniae* (n=14) and *E. coli* (=14) were the most common pathogens causing SSTIs, followed by *E. cloacae* (n=6), *M. morganii* (n=2) and *K. oxytoca* (n=2) and *E. aerogenes* (n=1), *C. freudii* (n=1), *Citrobacter braaki* (n=1) and *Serratia marcescens* (n=1). This is represented in Figure 19.

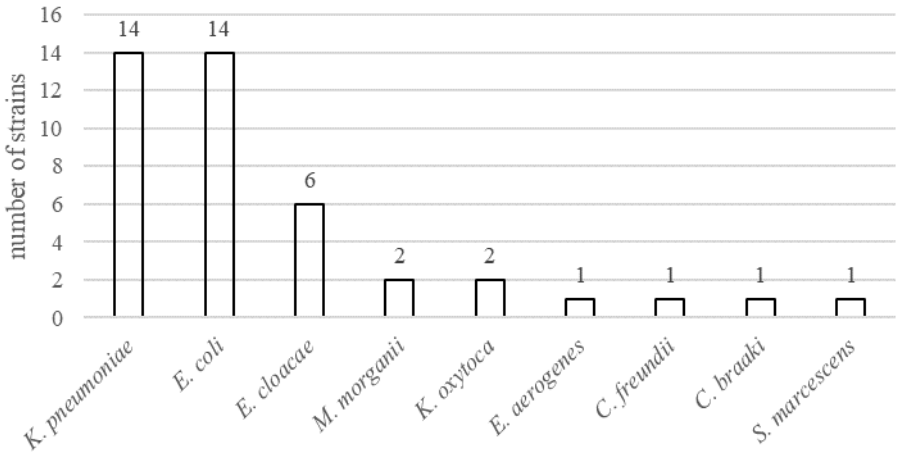


*Figure 19 - Distribution of different strains, that probably caused SSTIs, among all isolates tested positive for AmpC expression*

According to SENTRY Antimicrobial Surveillance Program, since 1998 to 2004, in Europe, the most microorganisms present in SSTIs are *S. aureus*, followed by *P. aeruginosa*, *E. coli*, *Enterococcus* spp., *Enterobacter* spp., coagulase-negative Staphylococci, β-Streptococcus, *Klebsiella* spp., *P. mirabilis* and at last *Acinetobacter* spp. So, the most Enterobacteriaceae found are *E. coli*, *Enterobacter* spp., *Klebsiella* spp., *P. mirabilis* and *Acinetobacter* spp. (Moet et al. 2007).

In the present study, the carriage of an AmpCs alone (n=25), both an AmpCs and an ESBLs (n=11) and both an AmpCs and CPOs (n=6) was analysed (Figure 20).

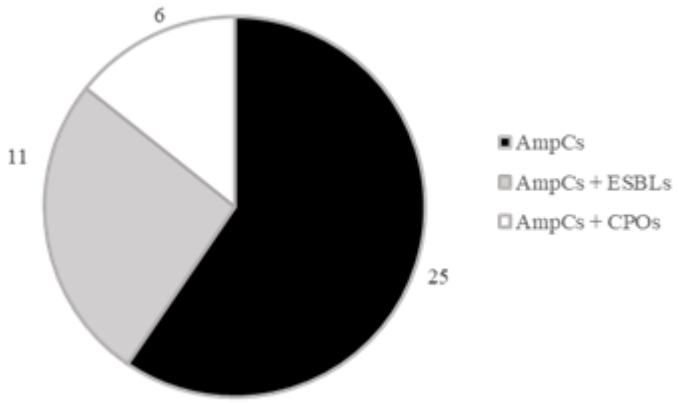


*Figure 20 - Distribution of combined resistance mechanisms among AmpC positive strains*

In this study, among 42 strains with AmpCs, 11 was confirmed to produce extended-spectrum β-lactases (ESBLs), namely, *K. pneumoniae* (7/11) and *E. coli* (4/11) (Figure 21).

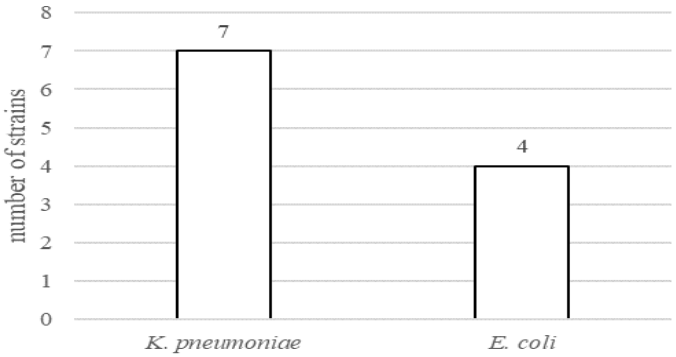


*Figure 21 - Distribution of strains producer both AmpCs and ESBL*

Several studies show that infections caused by ESBL-producing *E. coli* and *K. pneumoniae* have a significant impact on clinical outcome and are an emerging problem in ambulatory settings. For example, when was evaluated the effect of ESBL-producing *E. coli* and *K. pneumoniae* infection on clinical outcome, these were associated with a higher hospital rate (Caínzos 2008). The Infectious Diseases Society of America (IdSa) identified ESBL-producing

Enterobacteriaceae as problematic pathogens which will continue to compromise therapy for SSTIs (Moet et al. 2007; Eckmann and Dryden 2010).

In this study, among 42 Gram-negative strains collected with AmpCs, five *K. pneumoniae* and one *E. aerogenes* were resistant to carbapenems.

## 3. Conclusion

Urinary tract infections and skin and soft tissues infection are one of the causes of significant morbidity and mortality in the hospitals, leading to an economic burden (Caínzos 2008; Smirnov and Egbert 2016). In addition, an increased global prevalence of multi-drug resistant pathogens leads to complication in the antibiotic selection process. For examples, many times, the antimicrobial therapy is inappropriate when the infections are caused by a pathogen AmpC producer that lead to failure in treatments (Caínzos 2008; Eckmann e Dryden 2010; Conen et al. 2015).

The results obtained in the present study are worrisome since the species isolated are AmpC producer. These microorganisms have the ability to develop resistance during antimicrobial therapy with β-lactam antibiotics, like carbapenems and zwitterionic (sometimes referred to as fourth generation) cephalosporins (e.g., cefepime) (Thomson 2010). In addition, were observed that these infections caused by microorganisms are both nosocomial and non-nosocomial. This is more worrisome since the spread in community is higher.

In an attempt to solve this problem is of utmost importance choose new empirical regiments taking advantage of potent antibiotics or to develop new classes of antibiotics that are effective against these problems.

The control of the spread of multiply antibiotic-resistant pathogens is difficult. To improve it, laboratories should be able to detect AmpC β-lactamases since the diagnostic microbiology is essential to quality initiatives in hospitals. Furthermore, is necessary the development of new microbiological methods to simplify rapid and reliable detection of $bla_{\text{AmpC}}$ plasmid carriers.

Finally, is essential to continue monitoring these microorganisms to understand the trends in pathogens occurrences in hospitals and community and monitoring the resistance to antimicrobials commonly used. These programs, like SENTRY, are fundamental to modify the prescription locals guidelines and the infection control interventions if necessaire (Moet et al.

2007). This infection control can prevent additional infections and the spread of resistant pathogens (Thomson 2010).

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# Chapter III.

# Evaluate, *in vitro*, the activity of ceftolozane/ tazobactam against Enterobacteriaceae and *Pseudomonas aeruginosa*, which cause intra-abdominal infections

**Abstract:**

Ceftolozane/tazobactam (Ceft/Taz) is a new antibiotic resulting from the combination of a novel cephalosporin, structurally similar to ceftazidime, with tazobactam, a well-known β-lactamase inhibitor.

To evaluate the activity of this antimicrobial against Enterobacteriaceae and *Pseudomonas aeruginosa*, 44 non-duplicate Enterobacteriaceae and three *P. aeruginosa* clinical isolates were consecutively collected during the period from September to November 2018, from patients in Centro Hospitalar do Baixo Vouga with intra–abdominal infections.

Antimicrobial susceptibility testing was performed with the MIC Test Strip. Results were interpreted according to EUCAST criteria. Ceft/Taz showed good activity against almost all isolates (46/47), with MIC50 values comprising ≤0.38 and ≤4.00 μg/mL.

In conclusion, Ceft/Taz exhibited, in vitro, potent activity against Enterobacteriaceae and *P. aeruginosa*, including isolates that were multi-drug resistant, extended-spectrum β-lactamases producers, carbapenemases producers and AmpCs positive phenotype. Ceft/Taz has the potential to become a useful addition to the limited armamentarium of drugs that can be used to treat these problematic pathogens and has been recently approved for the treatment of complicated intra-abdominal infections.

**Keywords:** intra-abdominal infections, Enterobacteriaceae, *Pseudomonas aeruginosa*, AmpCs, extended-spectrum β-lactamases

## 1. Introduction

Intra-abdominal infections (IAIs) encompass a broad spectrum of pathological conditions, ranging from uncomplicated appendicitis to fecal peritonitis. According to the clinic, IAIs are subdivided into two main categories, uncomplicated and complicated (cIAIs) infections. Uncomplicated infections are located only in an organ and do not spread to the peritoneum, while cIAIs extends into more than one organ and causes peritonitis, which increases mortality rates (Sartelli et al. 2013; Eckmann and Solomkin 2015).

The most common pathogens involved in IAIs are Enterobacteriaceae, namely, *Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter* spp. and the treatment of these type of infections involves antimicrobial therapy (Maseda et al. 2014; Sartelli et al. 2014).

In mid-nineties, 95-97% of the microorganisms associated with IAIs were susceptible to commonly used antibiotics. However, microorganisms developed resistance against the drugs, due to excessive use of antimicrobials. For example, Gram-negative organisms exhibit resistance to β-lactams and co-resistance other antibiotics (Maseda et al. 2014).

Nowadays, it has been reported an increased number of MDR in IAIs and this type of infections are a global problem that requires utmost attention. IAIs caused by MDR were associated with longer duration of inadequate antimicrobial therapy and hospital length of stay, higher access to intensive care unit (ICU) and re-laparotomy. These factors lead to higher hospitalization costs, lower quality of care and increased morbidity and mortality. Recent exposure to antimicrobial agents (previous 90 days) was considered a risk factor for resistance. However, the presence of severe cardiovascular disease, leukocytosis or leukopenia, hospital-acquired infections, inadequate source control of the infection wasn't associated with risk of isolating MDR pathogens (Maseda et al. 2014; Labricciosa et al. 2018; Mohamed et al. 2018).

Ceftolozane/tazobactam (Ceft/Taz) is a new antibiotic resulting from the combination of a novel cephalosporin of the third generation with a β-lactamase inhibitor (Mohamed et al. 2018).

Ceftolozane (Figure 22) (previously CXA-101 and FR-264205) is structurally similar to ceftazidime. Ceftazidime is a third-generation cephalosporin and has been used for the treatment of Gram-negative infections for more than 20 years.

However, β-lactams, such as ceftazidime and cefotaxime, in presence of some PBPs are unstable since they increase the induction of AmpCs. So, at position 3 of the cephem nucleus of the new cephalosporin, the side-chain is modified (Moyá et al. 2010; Eckmann and Solomkin 2015; Giacobbe et al. 2018; Sebaaly et al. 2018; Sheu et al. 2018).

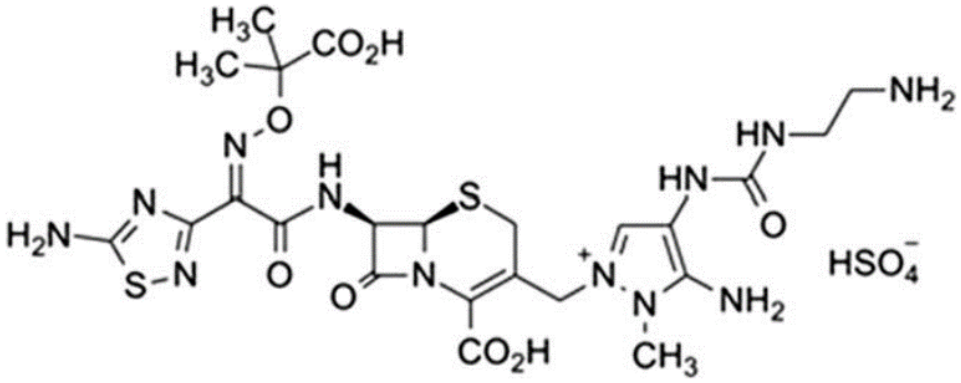


*Figure 22 - Chemical structure of ceftolozane. Adapted by (Sebaaly et al. 2018).*

A heavier chain, pyrazole, in ceftolozane, replaces the pyridinium found in ceftazidime. The presence of the pyrazole ring in ceftolozane gives it a steric hindrance, i.e., it prevents the binding of a novel binding, for example, β-lactamases. In this way, the hydrolysis of the compound is prevented, providing stability against the excessive production of AmpC β-lactamases produced by *P. aeruginosa*. In contrast, in case of ceftazidime, the pyridinium ring present confers a lower activity (Moyá et al. 2010; Eckmann and Solomkin 2015; Giacobbe et al. 2018; Sebaaly et al. 2018; Sheu et al. 2018).

In addition, ceftolozane have the aminothiadiazole ring at 7-position of the cephem nucleus of ceftolozane and that provide increased activity against Gram-negative bacilli when compared to other cephalosporins (Eckmann and Solomkin 2015).

Furthermore, ceftolozane inhibits penicillin-binding proteins (PBPs). For example, ceftolozane binds with high affinity to three essential PBPs of *E. coli* (PBP1a/b, PBP1b and PBP3) and also *P. aeruginosa*, inhibiting them (Eckmann and Solomkin 2015).

Like others oxyimino-cephalosporins, the activity of ceftolozane is compromised by extended-spectrum *β*-lactamases (ESBLs), depressed AmpC enzymes and carbapenemases (Livermore et al. 2010).

Tazobactam (Figure 23) is a well-known β-lactamase inhibitor. It inhibits most β-lactamases, including the most common ESBLs, such as CTX, SHV, TEM and the class C cephalosporinases. Tazobactam binds irreversibly to β-lactams and leads to slow hydrolysis. On the other hand, the ESBLs TEM-3–9, SHV-2–4, OXA-2, and CTX-M-3–18 reduce the activity of the tazobactam, but it may still remain effective (Sebaaly et al. 2018; Sheu et al. 2018).

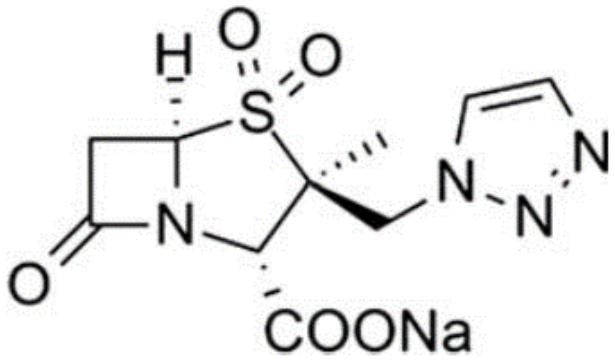


*Figure 23 - Chemical structure of tazobactam. Adapted by (Sebaaly et al. 2018)*

Therefore, the combination between ceftolozane and tazobactam increases the activity against many ESBL-producing Enterobacteriaceae and MDR *P. aeruginosa*. Ceft/Taz has the most potent antipseudomonal activity compared (Eckmann and Solomkin 2015).

Ceft/Taz exhibits excellent *in vitro* activity against Gram-negative microorganisms isolated from IAIs, UTIs and bloodstream infections, even when there are ESBL-producing microorganisms (Titelman et al. 2011; Ma and Hughes 2018; Sheu et al. 2018). For this reason, Ceft/Taz is an alternative to carbapenems for the treatment of infections caused by ESBLs microorganisms isolated from IAIs (Giacobbe et al. 2018; Sheu et al. 2018). For all the above, recently, Ceft/Taz has been approved for the treatment of cIAIs, by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (Giacobbe et al. 2018).

The main purpose of this study was to evaluate the *in vitro* activity of Ceft/Taz against ESBL-producing Enterobacteriaceae and MDR *P. aeruginosa* using a MIC test strip.

## 2. Results and Discussion

### 2.1. Bacterial strains characterization

During the period of this the study, a total of 47 microorganisms, including 44 Enterobacteriaceae and three *P. aeruginosa*, suspected to cause intra-abdominal infections were studied. As previously reported by other studies Enterobacteriaceae were found to be the most common bacteria involved in IAIs (Sartelli et al. 2014).

Among Gram-negative bacteria, the most frequent microorganism isolated was *E. coli* (24/47 total), followed by *K. pneumoniae* (10/24 total), *Enterobacter cloacae* (5/47) and *Pseudomonas aeruginosa* (3/47). The remaining strains, *Citrobacter freudii*, *Klebsiella oxytoca*, *Morganella morganii*, *Enterobacter aerogenes* and *Enterobacter asburiae* are present in the ration of 1/47 (Figure 24).

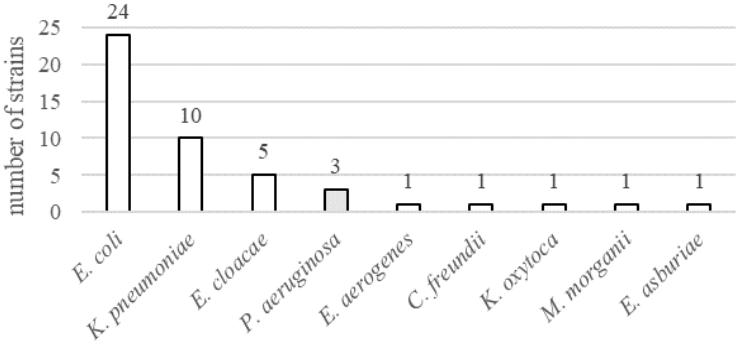


*Figure 24 - The different strains and our quantitative from IAIs*

Following the criteria described in Material and Methods section, 2, the microorganisms were classified as nosocomial or non-nosocomial. Overall 47 isolates collected, five were nosocomial in different wards of CHBV, whereas 42 were non-nosocomial (Figure 25). According to Sartelli et al. 2014, verified the same: 86.7% of patients were affected by community-acquired IAIs while the remaining 13.3% suffered from healthcare-associated infections.

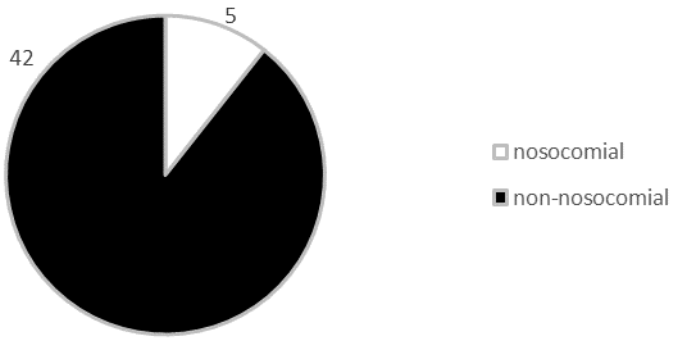


*Figure 25 - Distribution of the IAIs isolates considering if they were nosocomial and non-nosocomial*

In our study, all *P. aeruginosa* (n=3) were nosocomial. According to Sartelli et al. 2014, *P. aeruginosa* is one of the major nosocomial pathogens in the world.

### 2.1.1. Extended-Spectrum β-lactamases producing Enterobacteriaceae

Screen-positive for the ESBL phenotype was confirmed in 13 microorganisms; *E. coli* (5/24) and *K. pneumoniae* (8/10). These results are demonstrated in Figure 26.

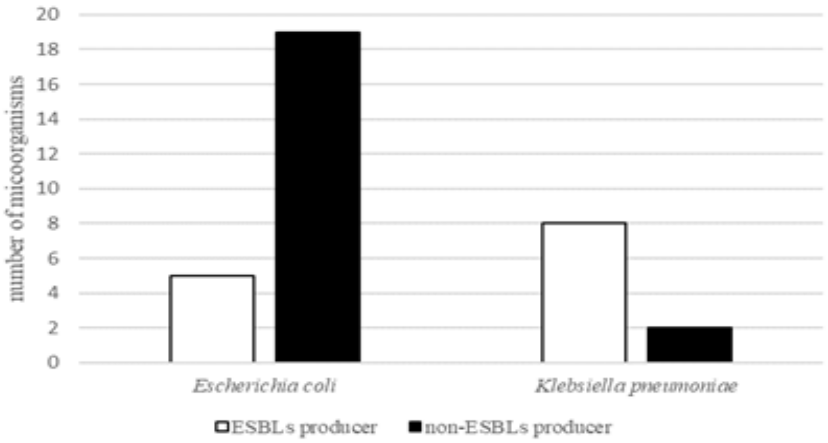


*Figure 26 - Number of ESBL-producing microorganisms; Escherichia coli (5/24) and Klebsiella pneumoniae (8/10)*

This is according to Sheu et al. 2018, which verified that *K. pneumoniae* and *E. coli* are the two most common species of the family Enterobacteriaceae ESBL producers.

Analysing the figure is evident that the number of ESBL-positive *K. pneumoniae* is higher than ESBL-positive *E. coli* isolates. These results are in accordance to those studies reported by Sheu et al. 2018, since ESBL-positive *K. pneumoniae* isolates comprised 42.8% of all identified *K. pneumoniae* isolates, while ESBL-positive *E. coli* isolates made up 20.6% of all identified *E. coli* isolates.

In this study, ESBL-positive Enterobacteriaceae were more prevalent in inpatients (10/13) than in outpatients (3/13), and this is according to Sartelli et al. 2014.

### 2.1.2. Multi-drug resistant Gram-negative

Among all isolates, eighteen were MDR organisms (18/47) were isolated, namely, *E. coli* (7/24), followed by *K. pneumoniae* (8/10), *E. cloacae* (2/5) and *M. morganii* (1/1). The remaining microorganisms (*P. aeruginosa*, *C. freudii*, *K. oxytoca* and *E. asburiae*) did not present multi-drug resistant phenotype as shown in Figure 27.

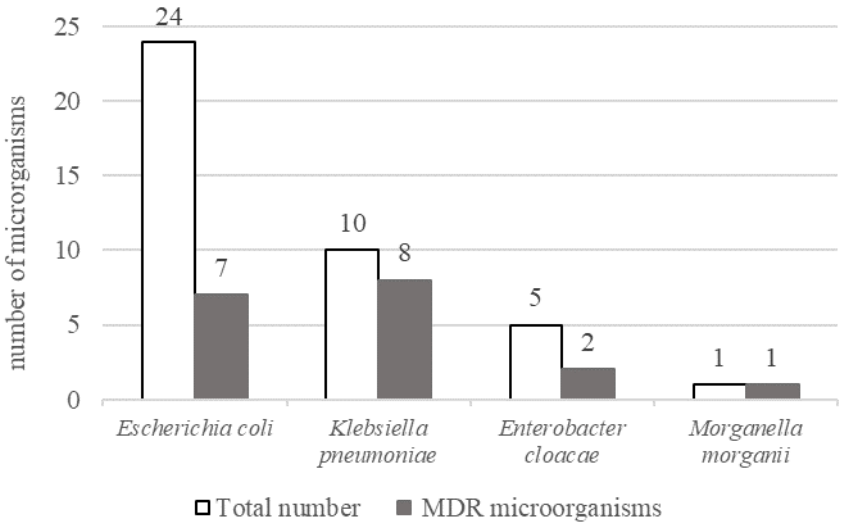


*Figure 27 - Strains of MDR microorganisms*

Fourteen MDR organisms were recovered from inpatients, while only four were recovered from outpatients. Our findings are in accordance with those previously described by Labricciosa et al. 2018, since, in this multi-center study, was found a significant difference between MDR organisms that were isolated from health-acquired and community-acquired infections, those being more frequent in the first group.

## 2.2. Antimicrobial susceptibility testing

From a total of 47 microorganisms, 46 were susceptible to Ceft/Taz and only one *K. pneumoniae* presented an intermediate phenotype. In other words, the overall susceptibility of *E. coli*, *E. cloacae*, *E. aerogenes*, *C. freundii*, *K. oxytoca*, *M. morganii*, *E. asburiae* and *P. aeruginosa* to ceftolozane/tazobactam was 100%.

Ceftolozane/tazobactam showed good activity against *E. coli* (n=24) with MIC50 values of 0.5 µg/ mL in 15 of 24 isolates tested and *K. pneumoniae* (n=10), with MIC50 values of 1.00 µg/ mL in four of ten isolates tested. Ceft/Taz also demonstrated good activity against other frequently isolated Enterobacteriaceae, such as, *E. aerogenes* (MIC50 1.50 µg/ mL, n=1), *C. freundii* (MIC50 0.38 µg/ mL, n=1), *K. oxytoca* (MIC50 0.50 µg/ mL, n=1), *M. morganii* (MIC50 0.50 µg/ mL, n=1), *E. cloacae* (MIC50 0.50 µg/ mL, n=5), *E. asburiae* (MIC50 0.50 µg/ mL, n=1); and *P. aeruginosa* (MIC50 0.75 µg/ mL, n=1; MIC50 1.00 µg/ mL, n=1; MIC50 1.50 µg/ mL, n=1). The MIC50 values for all strains isolated in IAIs showed in Table 4

The results of this study confirm previous studies was tested the activity of ceftolozane-tazobactam, *in vitro*, against *P. aeruginosa* (Shortridge et al. 2018).

*Table 4 - MIC50 values of susceptibility for Enterobacteriaceae (Enterobacter aerogenes, Esherichia coli, Citrobacter freudii, Klebsiella oxytoca, Morganella morganii, Klebsiella pneumoniae, Enterobacter cloacae and Enterobacter asburiae) and Pseudomonas aeruginosa*

| | MIC50 values of susceptibility for differents microorganisms (µg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0,38 | 0,50 | 0,75 | 1,00 | 1,50 | 2,00 | 3,00 | 4,00 |
| *Enterobacter aerogenes* | - | - | - | - | 1 | - | - | - |
| *Escherichia coli* | 4 | 15 | 2 | 1 | - | 1 | 1 | - |
| *Citrobacter freundii* | 1 | - | - | - | - | - | - | - |
| *Klebsiella oxytoca* | 1 | - | - | - | - | - | - | - |
| *Morganella morganii* | - | 1 | - | - | - | - | - | - |
| *Klebsiella pneumoniae* | 1 | 1 | 1 | 4 | 1 | 1 | - | 1 |
| *Enterobacter cloacae* | - | 5 | - | - | - | - | - | - |
| *Enterobacter asburiae* | - | 1 | - | - | - | - | - | - |
| *Pseudomonas aeruginosa* | - | - | 1 | 1 | 1 | - | - | - |

### 2.2.1. Extended-Spectrum β-lactamases producing Enterobacteriaceae

From a total of 24 *E. coli*, five isolates were positive for ESBL phenotype, nonetheless, all were susceptible to Ceft/Taz, which demonstrated potent activity (≤0.50 and ≤3.00 µg/mL). All non-ESBLs phenotype isolates were inhibited with Ceft/Taz that exhibited a potent activity (≤0.38 and ≤2.00 µg/mL). These values were demonstrated in Table 5.

*Table 5 - MIC50 values of susceptibility for Escherichia coli isolate with ESBL screen-positive phenotype and non-ESBL phenotype.*

| | MIC50 values of susceptibility for differents microorganisms (µg/mL) | | | | | | |
|---|---|---|---|---|---|---|---|
| | ESBLs producer | | non-ESBLs producer | | | | |
| *Escherichia coli* | 0,50 | 3,00 | 0,38 | 0,50 | 0,75 | 1,00 | 2,00 |
| | 4 | 1 | 4 | 11 | 2 | 1 | 1 |

From a total of ten *K. pneumoniae*, eight isolates exhibited an ESBL phenotype (n=8), which were susceptible to Ceft/Taz (≤0.38 and ≤1.50 µg/mL). All isolates non-ESBL phenotype were inhibited by Ceft/Taz and the activity is ≤0.50 and ≤4.0 µg/mL. These values were demonstrated in Table 6.

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*Table 6 - MIC50 values of susceptibility for Klebsiella pneumoniae isolates with ESBL screen-positive phenotype and non-ESBL phenotype.*

| | MIC50 values of susceptibility for differents microorganisms (µg/mL) | | | | | | |
|---|---|---|---|---|---|---|---|
| | ESBLs producer | | | | | non-ESBLs producer | |
| *Klebsiella pneumoniae* | 0,38 | 0,75 | 1,00 | 1,50 | 2,00 | 0,50 | 4,00 |
| | 1 | 1 | 4 | 1 | 1 | 1 | 1 |

The results of this study confirm previous studies where the activity of Ceft/Taz, *in vitro*, against Enterobacteriaceae, including those with extended-spectrum β-lactamases, was tested (Shortridge et al. 2018).

### 2.2.2. Multi-drug resistant Gram-negative

In this study, seven *E. coli* and eight *K. pneumoniae* strains present multi-drug resistance phenotype.

When Ceft/Taz was tested against MDR *E. coli* isolates (7/24), they were all susceptible to this antimicrobial that demonstrated a potent activity (≤0.50 and ≤3.00 µg/mL). Also, all non-MDR *E. coli* isolates (n=17) were inhibited by Ceft/Taz, which once again exhibited a potent activity (≤0.38 and ≤2.00 µg/mL). These results are demonstrated in Table 7.

*Table 7 - MIC50 values of susceptibility for MDR and non MDR Escherichia coli isolates.*

| | MIC50 values of susceptibility for differents microorganisms (µg/mL) | | | | | | |
|---|---|---|---|---|---|---|---|
| | MDR | | | non-MDR | | | |
| *Escherichia coli* | 0,50 | 1,00 | 3,00 | 0,38 | 0,50 | 0,75 | 2,00 |
| | 5 | 1 | 1 | 4 | 10 | 2 | 1 |

Considering MDR *K. pneumoniae* isolates (8/10), all the isolates tested were susceptible to ceftolozane/tazobactam with MIC50 values ranging ≤0.38 and ≤2.00 µg/ mL. Also MDR *K. pneumoniae* isolates were positive for ESBL phenotype screen. All non-MDR *K. pneumoniae* isolates (n=2) were inhibited by Ceft/Taz and the MIC50 values ranged from ≤0.50 to ≤4.00 µg/ mL. These results were demonstrated in Table 8.

*Table 8 - MIC50 values of susceptibility for MDR and non MDR Klebsiella pneumoniae isolates.*

| | MIC50 values of susceptibility for differents microorganisms (µg/mL) | | | | | | |
|---|---|---|---|---|---|---|---|
| | MDR | | | | | non-MDR | |
| *Klebsiella pneumoniae* | 0,38 | 0,75 | 1,00 | 1,50 | 2,00 | 0,50 | 4,00 |
| | 1 | 1 | 4 | 1 | 1 | 1 | 1 |

It should be noted that all strains were AmpC–producing isolates. When tested, ceftolozane/tazobactam was active, *in vitro*, against these isolates, with MIC50 values of ≤0.38 and ≤4.0 µg/ mL.

## 3. Conclusion

Complicated intra-abdominal infections (cIAIs) remain an important cause of morbidity (Sartelli et al. 2013).

Prompt identification the pathogens is essential for clinical management, to provide empiric antimicrobial therapy. For example, the detections of MDRs, by rapid microbiologic tests, providing a result on the same day could be used for patients with IAIs. These tests should be considered when patients showing high-risk factors, such as, antimicrobial therapy administered with seven days before operation on hospital admission. This approach is useful to implement infection control measures quickly, minimize cross-transmission and allow antimicrobial optimization. The antimicrobial optimization may lead to decreased mortality rate, hospitalization time and hospitalization costs (Labricciosa et al. 2018).

The emergence of resistance among Gram-negative bacteria, especially Enterobacteriaceae ESBL producers, multi-drug resistant strains, and *P. aeruginosa* has been a cause for major concern. The problem of resistance left clinicians with few viable alternatives and its necessary the development of new drugs that show activity against resistant strains (Mohamed et al. 2018; Seifert et al. 2018; Shortridge et al. 2018).

Ceft/Taz was shown efficient against Enterobacteriaceae and *P. aeruginosa*, including some microorganism ESBL producer and MDR, since was effective against almost all isolates (46/47). However, it is necessary to use this antimicrobial with precaution in order to prevent the development of resistance by microorganisms.

# Chapter IV.

# Development of pedagogical materials for the antibiotic resistance awareness

**Abstract:** "O (ir)resistível mundo dos antibióticos", is an activity included in the Projeto de Estímulo à Mobilidade de Ideias (PEMI), an educational project that is based on the awareness of Science and Health issues, aimed at social transformation, developed by Association for World Innovation in Science and Health Education (AWISHE). It was a different class, adapted to the objectives of the discipline "Health and Environment", that took place in the Agrupamento de Escolas de Oliveirinha, for 14 students at risk of dropping out of school. Experimental activities were carried out with the objective of consolidating the acquired knowledge. With this action it was possible to promote the interest, awareness and formation of opinions and appropriate decisions regarding the use of antibiotics. The results of the questionnaires, corroborate this, since the results showed that the grades obtained were positive in more than 70 %.

**Key-words:** antibiotics, resistance, WHO, AWISHE, PEMI, alternative curricular course

## 1. Introduction

The discovery/production of antibiotics in the first half of the previous century and its subsequent introduction into the clinic was one of the greatest achievements of medicine (Van Hoek et al. 2011). The use of antimicrobial agents reduced morbidity and mortality in humans. In addition, it has allowed the treatment of hitherto incurable diseases, contributing to the increase of life expectancy of the human being. In the modern era of medicine, antibiotics have become necessary in medicine, as in organ transplants (Van Hoek et al. 2011; Khan et al. 2018).

The development of new antimicrobial families over the years has allowed us to believe that we could always stay ahead of pathogens (World Health Organization 2001).

Although the resistance is a natural and biological phenomenon, in the preceding decade, we saw an increase in antimicrobial resistance in pathogens. The prevalence of resistance varies between geographical regions and over time, but sooner or later resistance emerges to every antimicrobial (World Health Organization 2001; Khan et al. 2018).

Antibiotic resistance as one of the most important public health of the 21st-century, more than 700,000 people die around the world due to resistant infections. In some instances, resistance to antimicrobial agents is seriously compromising treatment outcome (World Health Organization 2001; Khan et al. 2018).

According to World Health Organization (WHO), resistant microorganisms are strains able to multiply in presence of drug concentrations higher than the concentrations in humans receiving therapeutic doses (Van Hoek et al. 2011).

Antimicrobial excessive use is the key driver of resistance. However, the inappropriate use of antimicrobial agents is, also, associated with the emergence of resistance: misuse due to lack of access and underuse due to lack of financial support to complete treatment courses (World Health Organization 2001).

For this reason, control the spread of resistance and improving use are a priority. Several recommendations have been proposed by WHO for these and many of the recommendations are based on education.

Although most antimicrobial use occurs in the community, the intensity of use in hospitals is far higher. Hospitals are therefore important in the containment of antimicrobial resistance. In this environment is crucial to develop approaches to improving the use of antimicrobials, reducing the incidence and spread of hospital-acquired infections.

For this, it is necessary to educate healthcare workers, prescribers, dispensers and general community.

In addition, all prescriber and distributor groups about the importance of proper use of antimicrobials, disease prevention, and infection control. From here it is important to encourage them to educate their patients about the use of antimicrobials and the importance of adherence to prescribed treatments.

Furthermore, educate patients and the general community on the appropriate use of antimicrobials, on the importance of measures to prevent infection (such as immunization, vector control, use of bednets, etc.), on simple measures that may reduce transmission of infection in the household and community (such as handwashing, food hygiene, etc.), encourage appropriate and informed health care seeking behaviour, on suitable alternatives to antimicrobials for relief of symptoms and discourage patient self-initiation of treatment, except in specific circumstances.

Education should be the basic tool for the development of individuals. Thus, the educational process must begin as early as possible in their lives. In order for children and young people to achieve levels of knowledge that enable them to make a difference in communities, including prevention and health care.

To promote this education in all areas, the WHO have the responsibilities, such as, raise awareness of the problems posed by antimicrobial resistance, promote the sharing of information about and understanding of resistance, provide strategic and technical guidance on interventions to contain resistance, assist Member States to implement these interventions, stimulate research

to address the knowledge gaps and improve understanding of antimicrobial resistance and to encourage research and development of new antimicrobial agents.

Some projects are already implemented in world for achieve these goals. For example, in 1998, the WHO with many partners, development a strategy to provide, for all Member States, a framework of interventions to stimulate the prevention of infection, to slow the emergence of resistance and to reduce the spread of resistant microorganisms, in order to reduce the impact of resistance on health and health care costs, while improving access to existing agents and encouraging the development of new agents.

The World Health Assembly (WHA) Resolution of 1998 urged Member States to develop measures to encourage appropriate and cost- effective use of antimicrobials. This requires attention in sence for prohibit the dispensing of antimicrobials without the prescription of a qualified health care professional, to improve practices to prevent the spread of infection and thereby the spread of resistant pathogens. In addition, strengthen legislation to prevent the manufacture, sale and distribution of counterfeit antimicrobials, the sale of antimicrobials on the informal market and to reduce the use of antimicrobials in food-animal production. Countries were also encouraged to develop sustainable systems to detect resistant pathogens, to monitor volumes and patterns of use of antimicrobials and the impact of control measures.

Since the WHA Resolution, many countries have expressed growing concern about the problem of antimicrobial resistance, and some have developed national action plans to address the problem (World Health Organization 2001).

In Portugal, one of these examples is AWISHE (Association for World Innovation in Science and Health Education).

This project has as main objectives realize training and awareness actions about Science and Health subjects, the development educational activities for children, youngsters and adults and a permanent link with educational and cultural programs. In addition, promote the development of national and international Collaborative Learning Communities and access to information, educational opportunities, training and development (AWISHE). One of the approaches developed by AWISHE is the PEMI (Projeto de Estímulo à Mobilidade de Ideias). PEMI is an educational project that aims to bring to schools a versatile, accessible, and fun yet rigorous scientific knowledge and has as its fundamental principle the conscientization of science and health through education. It is intended to rise awareness into students to the importance of avoiding risk behaviours, and to take actions that lead to a good quality of life, individual and community.

This project intends to fill a void of alternative resources for a group of students at risk of dropping out of school, with an Alternative Curricular Course. In this situation, there are students with no expectations of the future, with interests that are parallel to the school and, therefore, need training for the exercise of citizenship after compulsory schooling, making them informed citizens, responsible, and intervening in society. These students attend compulsory subjects, but with a focus on the outside world. Thus, in addition to the structural component (Portuguese and Mathematics) and the school offers in the vocational component (gardening, table service, event organization, restoration and image), the "Health and Environment" discipline is also proposed in the context of the sciences.

This project is being implemented in primary and secondary schools, where scientific lectures and practical activities related to them are given.

The team is composed of scientists from different areas, such as Microbiology, Anthropology, Medicine, Neurosciences, among others. In addition to the AWISHE team, researchers and physicians are involved in this project.

## 2. Material and methods

Considering all the above, one of the activities developed by PEMI was "O (ir)resistível mundo dos antibióticos".

This different class took place at the school E/B 2 3 Castro Matoso belonging to the Agrupamento de Escolas de Oliveirinha, for a class of 14 students at risk of dropping out of school, with an Alternative Curricular Course. To this end, hands-on activities were carried out in order to consolidate the knowledge acquired, without losing scientific objectivity and accuracy.

It is focused on the awareness and scientific dissemination of the great global health problem we face today: resistance to antibiotics. To do so, a brief approach to bacteria, different types of antibiotics, and how excessive and improper use has led to the development of resistance to antibiotics.

One of the activities developed by "O (ir)resistível mundo dos antibióticos" intended to show its constitution of a bacterium. To do so recreational illustrations of a bacterial cell the organelle was used, the students were supposed to match nomenclature and function.

It was also intended that students could distinguish Gram-positive and Gram-negative bacteria, using Gram staining. Representative coccus and bacillus schemes were used to allow

comparison with their microscopic form. For this, strains of *Staphylococcus aureus* and *Escherichia coli*, respectively, were used.

In addition, through the use of a time-line linking different classes of antibiotics and their discovery, it was possible to understand the appearance of antibiotics and resistance as a natural and biological phenomenon. It was also raised the awareness to the problem of excessive use of antibiotics, which leads to the rapid spread of resistance. In the course of this, it was shown how the different classes of antibiotics and their mechanism of action act on the bacterium.

At the end of the session, a questionnaire with six multiple-choice questions was applied to all students, testing the essential topics covered.

## 3. Results and Discussion

During the session, the students were participative and attentive, especially during hands-on activities. In addition, we find it difficult to convey some scientific terms. However, it is here, in this process of communication / education, that lies the real challenge, since sometimes it is not easy to translate into current language this knowledge.

The questionnaire distributed at the end of the session obtained 70% of the positive classifications, validating project application and student acceptance. However, there were also very negative outcomes: students with only one or two correct answers. Thus, the development of alternative methods of evaluation could show a greater receptiveness of the content addressed.

## 4. Conclusion

This class focused on the awareness and scientific dissemination of the great global health problem that we are facing today: resistance to antibiotics. In this work, we have been able to promote the interest, awareness and formation of opinions and appropriate decisions regarding antibiotics.

I think this experience has been enriching for students and teachers, as it was for me. Students were able to enjoy a lesson with a different methodology, including hands-on activities to captivate their attention, with different people to teach them about other subjects. With special emphasis on behaviors and actions that can be apprehended and easily replicated, so that all who receive them become part of a community conscious and proactive

The teachers were also able to learn a little about the subjects covered and share the lessons with other teams, in this case, microbiologists.

For me, as a speaker, this experience was very rewarding, since we were able to deliver knowledge and practices to the younger audience in an interactive way, favoring the connection between the scientific hypothesis and the world around them. For these kinds of problems, it is necessary to act upstream of the problem, and for this an effort has to be made to educate the population. This is achieved through the provision of scientifically correct information. In addition, this information must be presented in a simple enough way so that even the least literate citizen can understand what is being transmitted.

The PEMI team intends to expand this type of activity to regular teaching groups, covering other levels of education. In other words, in addition to the application in non-regular classes, PEMI is also a complement to the regular curriculum traditionally applied, promoting a differentiated and attractive perspective of the different topics covered, as well as hans-on activities that consolidate the knowledge acquired.

Considering the potential for growth and applicability, PEMI will continue to promote the inclusion of students from different contexts as well as promoters who will be part of it. The intervention of the PEMI is not intended to be differentiated from its capabilities, but rather inclusive and applicable to all types of classes.

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# Annexes

# ANNEX I: GRAM STAINING

Early in the history of bacteriology, the detection of bacterial cells in the tissues was difficult, since most staining methods used coloured the bacterial cells.

Christian Gram development a procedure able to differentiate microorganisms from tissue cells.

Nowadays, the Gram reaction is a classic technique, widely used in microbiology laboratories and is the first step in the identification of bacterial species, given the characteristic behaviour of these before the Gram method. This method allows to distinguish Gram-positive and Gram-negative bacteria, according to the constitution of the cell wall of the bacteria. Gram-positive bacteria have a thick layer of peptidoglycan, while Gram negative bacteria have a more complex cell wall and a thin layer of peptidoglycan. Gram-positive bacteria blush violet, while Gram negative blush red with safranin. This procedure also allows visualizing the morphology of bacteria and classifying them into cocci and Gram positive or negative bacilli.

Gram staining procedure:

1. Place a drop of microbial suspension on the slide and make a smear;
2. Dry and set by moderate heat;
3. Cover the smear with crystal violet solution and allow to stand for 1 minute;
4. Drain the dye and wash under running water;
5. Cover the smear with lugol solute and let it act for 1 minute, wash under running water;
6. Discourage with alcohol-acetone for 30 seconds and rinse with water;
7. Cover the smear with safranin for 30 seconds;
8. Drain the dye, wash with water and allow to air dry;
9. Observe the optical microscope with immersion objective (100x): a) Violet-stained bacteria are designated Gram-positive. b) Red stained bacteria are designated Gram-negative bacteria.

# ANNEX II: POSTER ECCMID 2019 – "SURVEILLANCE OF PLASMID-MEDIATED MCR-1 GENE IN HUMAN ISOLATES, IN AVEIRO, PORTUGAL"

**Abstract:**

Background: Bacterial infections continue to be one of the leading causes of morbidity and mortality worldwide. Excessive and imprudent use of antibiotics led to the increase of resistance narrowing the therapeutic options. Colistin belongs to the family of polymyxins, with broad-spectrum activity against Gram-negative bacteria, including most species of the family Enterobacteriaceae and it is considered the last resource for MDR or XDR phenotype therapy. The emergence of colistin resistance has caused great concern and resistance mediated by the plasmid-borne mcr-1 gene has been detected worldwide in Multidrug resistant (MDR) Enterobacteriaceae. The aim of this study was to evaluate the presence of this gene in strains from patients attending the Centro Hospitalar do Baixo Vouga, E.P.E., Portugal.

Materials/methods: All the isolates included in this study were non-duplicate and identified with the automated method VITEK2® (BioMèrieux, France), using the Gram-negative bacteria identification card, antimicrobial susceptibility testing (AST) was estimated and the advanced expert system (AES) suggestions were taken in consideration. The strains identified as susceptible to colistin were confirmed by MICRONAUT-S broth microdilution Colistin MIC test (Merlin Diagnostika). Polymerase chain reaction technique was used to screen for the presence of the colistin resistance plasmid mediated mcr-1 gene.

Results: During the timeframe of this study (2017-2018) a total of 6189 strains were isolated among them nine strains exhibited non-intrinsic resistance to colistin were collected (5 *Klebsiella pneumoniae*, 2 *Escherichia coli*, 1 *Pseudomonas aeruginosa* and 1 *Hafnia alvei*). Five strains were recovered inpatients and the other four were isolated from outpatient samples. Patients age range from 56 to 89 years-old. The mcr-1 gene was detected in one *Escherichia coli* strain.

Conclusions: The results of this study show that the mcr-1 gene was present, as previously described. However, it does not explain the resistance in found in all the strains included in this study. Further studies will be undertaken to screen for the presence of other genes. Since the prevalence rate of carbapenem resistant Enterobacteriaceae (CRE) has been increasing in our region, we highlight the importance of a Surveillance Program to monitor the spread of the plasmid-mediated colistin resistance genes into MDR Gram-negative bacteria.

**Poster:**

# *Surveillance of plasmid-mediated mcr-1 gene in human isolates, in Aveiro, Portugal*

**Liliana Azevedo[1], Ana Rita Silva[1], Patrícia Matos[2], Marta Tacão[3], Isabel Henriques[3,4], Elmano Ramalheira[2], Sónia Ferreira[2,5]**

1. Department of Biology, Aveiro, Portugal, 2. Clinic Pathology Department, Portugal, 3. Centre for Environmental and Marine Studies (CESAM), Aveiro, Portugal, 4. Department of Life Sciences Faculty of Science and Technology , Portugal, 5. Department of Medical Sciences, iBiMed, Portugal

## Introduction

Bacterial infections continue to be one of the leading causes of morbidity and mortality worldwide. Excessive and imprudent use, as well as, the lack of new antibiotics led to the increase of resistance narrowing the therapeutic options. Colistin belongs to the polymyxins family, with broad-spectrum activity against Gram-negative bacteria, including most species of the family Enterobacteriaceae and it has emerged as last resource for Multidrug-resistant (MDR) Gram-negative bacteria or Extensively drug-resistant bacteria (XDR) phenotype therapy.

The emergence of colistin resistance has caused great concern and resistance mediated by the plasmid-borne *mcr-1*, *mcr-3*, *mcr-4* and *mcr-5* genes has been detected worldwide in different MDR Enterobacteriaceae.

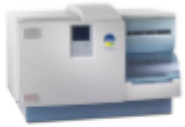
## Purpose

Evaluate the presence of ***mcr-1***, ***mcr-3***, ***mcr-4*** and ***mcr-5*** genes in strains from patients attending the Centro Hospitalar do Baixo Vouga, E.P.E., Portugal.

## Methods

Collection of strains during 2017-2019

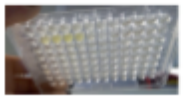
Culture plates



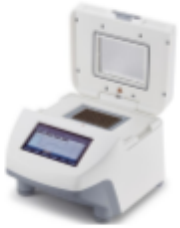
VITEK2®

Identification of 13 strains resistant to colistin

Confirmation of colistin resistance



MICRONAUT-S



Thermocycler CFX PCR

Screen for the presence of the *mcr-1*, *mcr-3*, *mcr-4* and *mcr-5* genes by PCR

## Results and conclusions


- Seven strains were recovered from inpatients and **six** from outpatient samples, whose age range from 56 to 89 years-old;
- The *mcr-1* gene was detected in one *Escherichia coli* strain, while *mcr-3*, *mcr-4* and *mcr-5* genes were not detected;
- The prevalence rate of carbapenem resistant Enterobacteriaceae has been increasing in our region, thus we highlight the importance of a Surveillance Program to monitor the spread of the plasmid-mediated colistin resistance genes into MDR Gram-negative bacteria.

## References

1. Poirel, Laurent, Jayol A, NP. Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes. Clinical Microbiology and Infection. 2017;30(2):557–96.

2. Tacão M, Tavares R dos S, Teixeira P, Roxo I, Ramalheira E, Ferreira S, et al. Mcr-1 and $bla_{KPC-3}$ in *Escherichia coli* sequence type 744 after meropenem and colistin therapy, Portugal. Emerging Infectious Diseases. 2017;23(8):1419–21.

# ANNEX III: POSTER SCICOM PT 2019 - MOBILIDADE DE IDEIAS NO CAMINHO DA INCLUSÃO

**Abstract:**

O Projeto de Estímulo à Mobilidade de Ideias (PEMI) é um projeto educacional desenvolvido pela Association for World Innovation in Science and Health Education (AWISHE), e é impulsionado pelo princípio fundamental desta associação: a consciencialização para assuntos de Ciência e Saúde através da Educação. Do portfolio da AWISHE fazem parte outros programas de comunicação de Ciência, nomeadamente projetos internacionais de formação de professores e profissionais de Saúde, sempre com foco na Saúde.

O PEMI, a ser implementado em escolas do ensino básico e secundário, dinamiza palestras de carácter científico, como veículo de informação entre a comunidade científica e as escolas, que incluem atividades práticas hands-on para consolidar os temas abordados em cada sessão.

Dentro das diferentes áreas que este projeto leva às escolas, são abordados temas de interesse aos jovens em idade escolar, com especial ênfase em comportamentos e ações que podem ser apreendidos e facilmente replicados, para que todos quantos os recebam passem a fazer parte de uma comunidade consciente e pró-ativa.

Até à data, a equipa do PEMI é constituída por investigadores, médicos e enfermeiros, com a missão de levar às escolas conhecimento científico rigoroso e versátil, bem como acessível e divertido. A equipa está disponível para complementar a matéria do professor com momentos de partilha de conhecimentos e boas práticas, de uma forma interativa, favorecendo a ligação entre o manual escolar, a hipótese científica e o mundo que nos rodeia.

Na génese deste projeto esteve a necessidade de preencher um vazio de recursos alternativos para uma turma de alunos em risco de abandono escolar, com um Percurso Curricular Alternativo. Nesta situação, estão alunos sem expectativas de futuro, com interesses paralelos à escola, e que necessitam de formação para o exercício da cidadania após a escolaridade obrigatória, tornando-os cidadãos informados, responsáveis, e intervenientes na sociedade. No entanto, a intervenção do PEMI não pretende ser diferenciadora das suas capacidades, mas antes inclusiva e aplicável a todo o tipo de turmas. Os temas oferecidos vão de encontro a grande parte do currículo escolar, tanto do ensino regular como dos currículos alternativos oferecidos pelas escolas.

Numa primeira fase, o PEMI foi implementado, ao longo do 2º período do ano letivo 2018/2019, no Agrupamento de Escolas de Oliveirinha, numa turma de Percurso Curricular Alternativo, com 25 alunos. A estes alunos é pedido que frequentem disciplinas de carácter obrigatório, mas numa vertente mais social e menos teórica. No contexto das Ciências, uma das disciplinas propostas é a disciplina "Saúde e Ambiente", onde se integrou o PEMI, sensibilizando os alunos para a importância de evitar comportamentos de risco, e assumir ações que levem a uma boa qualidade de vida, individual e comunitária. No final de cada sessão, foi aplicado a todos os alunos um questionário de escolha múltipla, testando os tópicos essenciais abordados. Até à data, as classificações foram positivas em mais de 50% dos alunos envolvidos, validando aplicação do projeto e aceitação por parte dos alunos.

Para além da aplicação em turmas de ensino não regular como esta, o PEMI constitui também um complemento ao currículo regular. Considerando o potencial de crescimento e aplicabilidade, o PEMI vai continuar a promover a inclusão, tanto de alunos de diferentes contextos, como de promotores que dele vierem a fazer parte.

**Poster:**

# PEMI: Mobilidade de ideias no caminho da inclusão

**Inês Cravo Roxo[1,2], Ana Santos-Carvalho[1,2], Ana Rita Silva[3], Daniela Meireles[4], João Borges[4], Joel Pinto[4], Liliana Azevedo[3], Patrícia Matos[3], Patrícia Quitério[4], Paulo Almeida[4], Rafaela Araújo[4], Richard Marques[1,5], Rui Soares[1,6,7], Susana Alarico[1,2], Sónia Ferreira[1,3,4]**

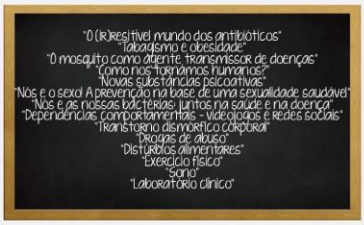
1 - Association for World Innovation in Science and Health Education (AWISHE); 2 - Centro de Neurociências e Biologia Celular, Universidade de Coimbra (CNC-UC); 3 - Universidade de Aveiro (UA); 4 - Centro Hospitalar do Baixo Vouga, Aveiro (CHBV); 5 - Grupo de Estudos em Evolução Humana (GEEvH); 6 - Instituto Português de Oncologia de Coimbra (IPO); 7 - Faculdade de Medicina da Universidade de Coimbra (FMUC).


O Projeto de Estímulo à Mobilidade de Ideias (**PEMI**) é um projeto educacional desenvolvido pela Association for World Innovation in Science and Health Education (**AWISHE**), segundo o princípio fundamental desta associação: a consciencialização para assuntos de Ciência e Saúde através da Educação. Do portefólio da AWISHE fazem parte outros programas de comunicação de Ciência, nomeadamente projetos internacionais de formação de professores dos ensinos básico e secundário, bem como profissionais de Saúde.

A intervenção do PEMI nas escolas pretende ser uma ação inclusiva e aplicável a todo o tipo de turmas e currículos escolares, favorecendo o desempenho global dos alunos sob a forma de complemento aos programas estabelecidos.

## Palestras de carácter científico rigoroso e atividades práticas divertidas

- Dinamização de palestras de carácter científico, como veículo de informação entre a comunidade científica e as escolas, que incluem atividades práticas *hands-on* para consolidar os temas abordados em cada sessão.
- Temas de interesse aos jovens em idade escolar, com especial ênfase em comportamentos e ações que podem ser apreendidos e facilmente replicados, para que todos quantos os recebam passem a fazer parte de uma comunidade consciente e pró-ativa.




## Equipa multidisciplinar

- A equipa do PEMI é constituída por investigadores, médicos e enfermeiros.
- Equipa disponível para complementar a matéria do professor com momentos de partilha de conhecimentos e boas práticas, de uma forma interativa, favorecendo a ligação entre o manual escolar, a hipótese científica e o mundo que nos rodeia.

## Complemento ao ensino regular e estratégia para currículos alternativos

- Na génese deste projeto esteve a necessidade de preencher um vazio de recursos alternativos para uma turma de alunos em risco de abandono escolar, com um Percurso Curricular Alternativo, de forma a contornar as baixas expectativas de futuro e interesses paralelos à escola.
- O PEMI constitui também um complemento ao currículo regular. Considerando o potencial de crescimento e aplicabilidade, o PEMI vai continuar a promover a inclusão, tanto de alunos de diferentes contextos, como de promotores que dele vierem a fazer parte.

## Comunidades de Aprendizagem Colaborativa

- Formação conducente ao exercício da cidadania após a escolaridade obrigatória.
- Sensibilização dos alunos para a importância de evitar comportamentos de risco, e assumir ações que levem a uma boa qualidade de vida, individual e comunitária.
- Estímulo social que resulta em cidadãos informados, responsáveis, e intervenientes numa sociedade inclusiva.

## Resultados e perspetivas futuras

- Primeira fase – ao longo do 2º período do ano letivo 2018/2019, no Agrupamento de Escolas de Oliveirinha, para uma turma de Percurso Curricular Alternativo, com 25 alunos, com avaliação por questionário individual de escolha múltipla, analisando a apreensão dos conteúdos apresentados: classificação média de 60% de respostas corretas.
- Execução das atividades práticas respetivas com sucesso, boa recetividade, e reprodutibilidade de conceitos *a posteriori*.
- No seguimento do sucesso da aplicação do Projeto numa turma de currículo alternativo no Agrupamento de Escolas de Oliveirinha, encontra-se a decorrer a segunda edição do PEMI dirigido a uma turma de 30 alunos do ensino regular do Colégio Maria Auxiliadora, em Canoas, Rio Grande do Sul, Brasil.


Para uma comunidade consciente e interventiva

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# ANNEX IV: POSTER SCICOM PT 2019 – "O (IR)RESISTÍVEL MUNDO DOS ANTIBIÓTICOS"

This poster was classified as the "2nd best poster" of the SciCom2019 congress, held in Aveiro.

**Abstract:**

"O (ir)resistível mundo dos antibióticos", é uma atividade enquadrada no Projeto de Estímulo à Mobilidade de Ideias (Projeto da Escola da Mãe da Inês, PEMI), um projeto educacional que tem por base a consciencialização de assuntos de Ciência e Saúde, visando a transformação social, desenvolvido pela Association for World Innovation in Science and Health Education (AWISHE).

Esta aula diferente, adaptada aos objetivos da disciplina "Saúde e Ambiente", ocorreu no Agrupamento de Escolas de Oliveirinha, para uma turma de cerca de 20 alunos em risco de abandono escolar, com um Percurso Curricular Alternativo.

Incidiu na consciencialização e divulgação científica do grande problema de saúde global com o qual nos deparamos hoje em dia: a resistência aos antibióticos. Para tal, iniciou-se uma breve abordagem às bactérias, aos diferentes tipos de antibióticos e de que forma o seu uso excessivo levou ao desenvolvimento de resistência aos mesmos.

O discurso foi ajustado às especificidades do público-alvo, dinamizando uma aula de carácter científico, recorrendo a atividades lúdicas, motivando e captando a audiência, por forma a consolidar o conhecimento adquirido foi consolidado, sem nunca perder a objetividade e rigor científicos.

Das atividades experimentais e demonstrações realizadas salientamos a visualização, ao microscópio ótico, das diferenças pela coloração de Gram, e a realização de uma linha temporal para a compreensão da ligação entre o uso exagerado de antibióticos e a sua resistência, entre outras.

Espera-se com este tipo de ação conseguir promover o interesse, sensibilização e formação de opiniões e decisões apropriadas, relativamente ao uso de antibióticos.

**Poster:**

# O (ir)resistível mundo dos antibióticos

**Ana Rita Silva[1] | Liliana Azevedo[1] | Patrícia Matos[2] | Inês Cravo Roxo[3,4] | Sónia Ferreira[2,3,5]**

1. Departamento de Biologia, Universidade de Aveiro; 2. Centro Hospitalar do Baixo Vouga, E.P.E., Portugal; 3. Association for World Innovation in Science and Health Education (AWISHE); 4. Centro de Neurociências e Biologia Celular, Universidade de Coimbra; 5. iBiMED - Instituto de Biomedicina, Universidade de Aveiro.

## INTRODUÇÃO

"O (ir)resistível mundo dos antibióticos" é uma atividade enquadrada no PEMI, um projeto educacional desenvolvido pela AWISHE, constituído por uma equipa de investigadores, médicos e enfermeiros. Este projeto promoveu a partilha de informação entre a comunidade científica e uma turma de alunos em risco de abandono escolar, com um Percurso Curricular Alternativo, visando a transformação social.

A atividade teve por base a consciencialização de assuntos de Ciência e Saúde, recorrendo à educação e divulgação científica do atual e preocupante problema de saúde global: a resistência a antibióticos.

## BACTÉRIAS

Através de ilustrações recreativas de uma célula bacteriana pretendeu-se mostrar a sua constituição. Para tal, fez-se corresponder cada organelo à sua respetiva nomenclatura e função.

## VISUALIZAÇAO AO MICROSCÓPIO ÓTICO

O método de coloração de Gram permitiu distinguir bactérias de Gram-positivo e de Gram-negativo, recorrendo a estirpes de *Staphylococcus aureus* e *Escherichia coli*, respetivamente. Posteriormente, compararam-se as suas formas microscópicas com exemplares macroscópicos.

## LINHA DE TEMPO

Mediante a utilização de uma linha de tempo onde se interligaram diferentes classes de antibióticos e a sua descoberta, pretendeu-se demonstrar que o aparecimento destes e a correspondente resistência é um fenómeno natural e biológico. Alertou-se, também, para o uso exagerado e inapropriado de uma terapia antimicrobiana que culmina numa disseminação rápida e global da resistência.

Todas as atividades tiveram na sua génese um discurso ajustado às especificidades do público-alvo, incluindo atividades práticas de forma a consolidar o conhecimento adquirido, sem nunca perder a objetividade e rigor científicos.

## RESULTADOS E DISCUSSÃO

Aquando da sessão, os alunos mostraram-se participativos e atentos. No final, foi distribuído um questionário de escolha múltipla cujas 70% das classificações foram positivas. Porém, o desenvolvimento de métodos alternativos de avaliação poderia evidenciar uma maior recetividade dos conteúdos abordados.

## CONCLUSÃO

A equipa pretende expandir este tipo de atividades a turmas de ensino regular, abrangendo outros níveis de escolaridade. Espera-se, assim, sensibilizar os cidadãos de modo a despertar o seu interesse e desencadear opiniões e decisões apropriadas, tornando-os parte de uma comunidade mais consciente e pró-ativa.

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# ANNEX V: ABSTRACT OF ICID 2020– EPIDEMIOLOGY OF CARBAPENEMASES-PRODUCING BACTERIA IN CENTRO HOSPITALAR BAIXO VOUGA

**Abstract:**

Infectious diseases caused by bacteria are one of the major health problems. The emergence of carbapenemases such as KPC, which have a high capacity of dissemination, increased this problem thus being motive of concern. The aim of this study was to evaluate the presence of KPC gene and their distribution in strains collected in Centro Hospitalar do Baixo Vouga, E.P.E., Portugal.

All isolates included in this study are non-duplicate and were identified using Gram-negative bacteria identification card of the automated system VITEK2® (BioMèrieux, France), antimicrobial susceptibility testing (AST) was estimated and advanced expert system (AES) rules were taken in consideration. CarbaNP (BioMèrieux, France) test was used, according to manufacture's instructions, to confirm the presence of a carbapenemase. Strains with a positive result were further studied by PCR, to confirm the presence of KPC gene and divided into different clusters. In addition, a PCR scheme was designed to detect the distribution of $bla_{KPC}$-harboring IncFIA (pBK30661-like and pBK30683-like) plasmids when they present the KPC gene.

During the timeframe of this study (2017-2018) a total of 6189 strains were isolated among them 61 Enterobacteriaceae and 3 *Pseudomonas aeruginosa* exhibited resistance to the carbapenems. Among all the isolates *Klebsiella pneumoniae* was the most relevant species since 52 strains were identified. The remain isolates were: five *Enterobacter cloacae*, one *Escherichia coli*, one *Citrobacter freundii* and one *Enterobacter aerogenes*. The PCR was positive for the presence of the KPC gene in 50 of 52 strains, which were all *Klebsiella pneumoniae*. The strains of *Klebsiella pneumoniae* are divided into 26 clusters, *Enterobacter cloacae* into 4 different clusters and *Pseudomonas aeruginosa* into 2 clusters by BOX-PCR. KPC-harboring IncFIA plasmids were found in 27 of 52 *K. pneumoniae* isolates.

The results of this study show that in our region, as expected, the KPC gene was prevalent in the *Klebsiella pneumoniae* and the majority was transferred in IncFIA plasmid. Nonetheless the percentage of carbapenems resistant Enterobacteriaceae being low (1%), it's

extremely important to apply earlier diagnostic techniques, such as molecular techniques, that will help to contain the spread more rapidly.