Universidade de Aveiro
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Inês Margarida Novais Ferreira

EPIDEMIOLOGY OF β-LACTAMASES PRODUCING STRAINS: ESBL, KPC AND OXA-48

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

o júri

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palavras-chave Resistência a antibióticos, antibióticos β-lactâmicos, epidemiologia, β-lactamases, *K. pneumoniae*, KPC, OXA-48.

resumo

A descoberta dos antibióticos foi um momento muito importante na história da humanidade, pois a comunidade de saúde viu uma solução para o tratamento das doenças infeciosas. No entanto, com o aumento do uso de antibióticos também aumentou a resistência aos mesmos, e consequentemente a dificuldade de tratar infeções causadas por microrganismos multirresistentes (MDR), tanto em ambiente hospitalar como na comunidade.

O principal objetivo deste estudo passa por avaliar a epidemiologia da resistência aos β-lactâmicos, nomeadamente os principais mecanismos de resistência presentes em estirpes de Enterobacteriaceae resistentes aos carbapenemos (ERC) de utentes do Centro Hospitalar do Baixo Vouga E.P.E (CHBV), entre abril de 2019 e fevereiro de 2020. Além disso, foi também desenvolvido um protocolo de extração de ácidos nucleicos com o intuito de deteção de genes de resistência de interesse no laboratório de microbiologia do CHBV.

Os resultados obtidos demonstram a presença da *Klebsiella pneumoniae* carbapenemase (KPC) em 43 dos 52 isolados resistentes a carbapenemos analisados, e maioritariamente em estirpes de *K. pneumoniae* (38/52). Para além disto, foi também detetada a presença de duas estirpes produtoras de OXA-48 em ambiente hospitalar, bem como a co-ocorrência da resistência a carbapenemos com a produção de outras β-lactamases, nomeadamente AmpCs e ESBL.

Em suma, este trabalho enfatiza a emergência de estirpes resistentes em diferentes ambientes, mas principalmente a nível hospitalar. Assim, é notória a necessidade e urgência de monotorização e controle epidemiológico em relação às estirpes multirresistentes, bem como a sensibilização em relação ao grave problema da resistência aos antibióticos entre a comunidade geral.

keywords Antibiotic resistance, β-lactam antibiotics, epidemiology, β-lactamases, *K. pneumoniae*, KPC, OXA-48.

abstract

The discovery of antibiotics was a turning point in human history, as the health community saw a solution for the treatment of infectious diseases. However, with the increased use and misuse of antibiotics, resistance to them has also increased, and consequently the difficulty in treating infections caused by multi-drug resistant (MDR) microorganisms, both in the hospital settings and the community.

The aim of this study was to evaluate the epidemiology of resistance to β-lactams, namely the main resistance mechanisms present in strains of carbapenem-resistant Enterobacteriaceae (CRE) from patients attending the Centro Hospitalar do Baixo Vouga E.P.E. (CHBV), between April 2019 to February 2020. In addition, a nucleic acid extraction protocol was also developed in order to detect resistance genes of interest in the CHBV microbiology laboratory.

The results obtained demonstrated the presence of *Klebsiella pneumoniae* carbapenemase (KPC) in 43 of the 52 carbapenem-resistant isolates analyzed, and mostly in strains of *K. pneumoniae* (38/52). In addition, the presence of two nosocomial OXA-48 producing strains was also detected, as well as the co-occurrence of resistance to carbapenems with the production of other β-lactamases, namely AmpCs and ESBL.

In short, this work emphasizes the emergence of resistant strains in different environments, but mainly at the hospital environment. Thus, there is a clear need and urgency for continued surveillance and epidemiological monitoring regarding MDR strains, as well as awareness of the serious problem of antibiotic resistance among the general community.

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Abbreviations

AmpC – Ampicillin hydrolysing enzyme
AROs – Antimicrobial-resistant organisms
AST – Antimicrobial susceptibility test
CDC – Centers for Disease Control and Prevention
CPE – Carbapenemase-producing Enterobacteriaceae
CPOs – Carbapenem-resistant organisms
CRE – Carbapenem-Resistant Enterobacteriaceae
DNA – Deoxyribonucleic acid
EAAD – European Antibiotic Awareness Day
ECDC – European Centre for Disease Prevention and Control
EIDs – Emerging infectious diseases
ESBLs – Extended spectrum β-lactamases
EU/EEA – European Union and European Economic Area
EUCAST – European Committee on Antimicrobial Susceptibility Testing
HCAIs – Health-care associated infections
HGT – Horizontal gene transfer
HICs – High-income countries
HIP – Hospital Infante D. Pedro
IAIs – Intraabdominal infections
IMP – Imipenemase metallo-β-lactamase
KPC – *Klebsiella pneumoniae* carbapenemase
LMICs – Low- and middle-income countries
MBL – Metallo-β-lactamase
MDR – Multi-drug resistant
MGEs – Mobile genetic elements
MIC – Minimal inhibitory concentration
MRSA – Methicillin-Resistant *Staphylococcus aureus*
NDM – New Delhi MBL
PBPs – Penicillin-binding protein
RNA – Ribonucleic acid
UTIs – Urinary tract infections
VIM – Verona integron-encoded metallo-β- lactamase
WAAW – World Antimicrobial Awareness Week
WHO – World Health Organization

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General Introduction

1. Bacterial infections

Since the discovery of bacterial infectious agents in the late 19th century, bacterial infections have been a major cause of disease and currently continue to be a big cause of mortality and morbidity worldwide (Davies and Davies, 2010; Reygaert, 2018).

Emerging infectious diseases (EIDs) have been increasing since 1940, with their peak incidence in the 1980s (Jones *et al.*, 2008). Although the majority of recent EIDs have been caused by viral pathogens, bacterial infections are also a threat, especially due to antimicrobial-resistant organisms (AROs), whose global spread has recently been identified by the World Health Organization (WHO), the European Union, the U.S. Government, and the Centers for Disease Control and Prevention (CDC) as one of the most significant threats to human health (Bloom, Black and Rappuoli, 2017; Pitout *et al.*, 2020).

Health-care associated infections (HCAIs) or nosocomial infections are infections acquired by patients at a health facility and are the most common complications affecting hospitalized patients. Some of the risk factors for these infections are patients who already have contagious diseases, health professionals, medical procedures, surgeries, and even antibiotic treatment. Urinary tract infections (UTIs) (usually catheter-associated), surgical-site infections, bloodstream infections (usually associated with the use of an intravascular device), and pneumonia are the major types of infections regarding nosocomial infections, and normally are associated with multiple pathogenetic pathways (Jones *et al.*, 2008; Madigan *et al.*, 2016).

1.1. Prevalent microorganisms associated with infections

Currently, the most notorious superbug is the Gram-positive organism *Staphylococcus aureus*. It is carried as a nasal commensal in 30% of the population, and its presence has long been linked to common skin infections. The landmark discovery and introduction of methicillin antibiotic in 1959 were thought to be a sure defence against penicillinases, but then appeared the methicillin-resistant *S. aureus* (MRSA). Recently, MRSA has moved outside the hospital and become a major community-acquired pathogen (Davies and Davies, 2010). Other Gram-positive bacteria associated with HCAIs are *Clostridium difficile* and *Streptococcus* spp. (Morehead and Scarbrough, 2018).

Regarding Gram-negative pathogens, *Pseudomonas aeruginosa* has evolved from being a burn wound infection into a major nosocomial threat. *Acinetobacter baumannii* is a more recent Gram-negative pathogen and is also primarily nosocomial (Davies and Davies, 2010).

Furthermore, members of the Enterobacteriaceae family are found worldwide and are known for causing a variety of diseases in humans (and animals), including intraabdominal infections (IAIs), UTIs, ventilator-associated pneumonia, wound and burn infections and bacteremia (Weinstein, Gaynes and Edwards, 2005; Kaye and Pogue, 2015). *Escherichia coli*, *Enterococcus* spp., *Klebsiella pneumoniae*, *Enterobacter* spp., *Proteus* spp., and *Salmonella enterica* are some of the most prevalent microorganisms found in healthcare facilities (Peleg and Hooper, 2012).

2. Antibiotics

Bacterial infections have been a major cause of disease and currently continue to be a big cause of mortality and morbidity worldwide (Reygaert, 2018). Antibiotics are substances that exhibit selective toxicity, they can have a bactericidal effect, causing the death of bacteria, or a bacteriostatic effect, inhibiting bacterial growth of pathogenic microorganisms without affecting the host. For that reason, antibiotics are the compounds of choice for the treatment and prevention of bacterial infections and thanks to them countless human lives have been saved (Ferreira, Sousa and Lima, 2010; Madigan *et al.*, 2016; Sultan *et al.*, 2018).

The first antibiotics were substances produced by microorganisms that prevented the growth of other microorganisms, which is exactly what Sir Alexander Fleming observed when he discovered penicillin in 1928: the inhibition of the growth of *S. aureus* due to the presence of the fungus *Penicillium notatum* (Sultan et al., 2018). Antibiotics can be isolated from many different microbial sources. Nonetheless, should be noted that the compounds referred to as antibiotics are generally present at undetectably low concentrations in the environment and that very few convincing demonstrations of *in situ* antibiotic activity have been reported (Davies, 2014). Nowadays, antibiotics are substances that are naturally produced by fungi, plants, bacteria, or chemically synthesized in laboratory (Ferreira, Sousa and Lima, 2010).

Antibiotics can be categorized in several ways, as they differ from each other based on their physical, chemical, pharmacological properties, spectrum and mechanism of action (Madigan *et al.*, 2016). They usually are classified based on their structure and degree of affinity with the target sites, and the main antibiotic classes are aminoglycosides, β-lactams, tetracyclines, macrolides, sulfonamides, quinolones, glycopeptides and polymyxins (Chellat, Raguž and Riedl, 2016).

Although penicillin was discovered in 1928, was only placed on the market in 1940, following the development of a method for its industrial production. Before that, in 1935, an antibacterial agent, precursor of the first sulfonamide, named Prontosil, began to be used against systemic

bacterial infections. A few years later, with the beginning of industrial production of penicillin G (benzylpenicillin) began the golden age of antibiotic discovery, mainly between the 1940s and 1970s, where all antibiotics that are currently known have been discovered and consequently introduced into clinical practice (figure 1) (Durand, Raoult and Dubourg, 2019).

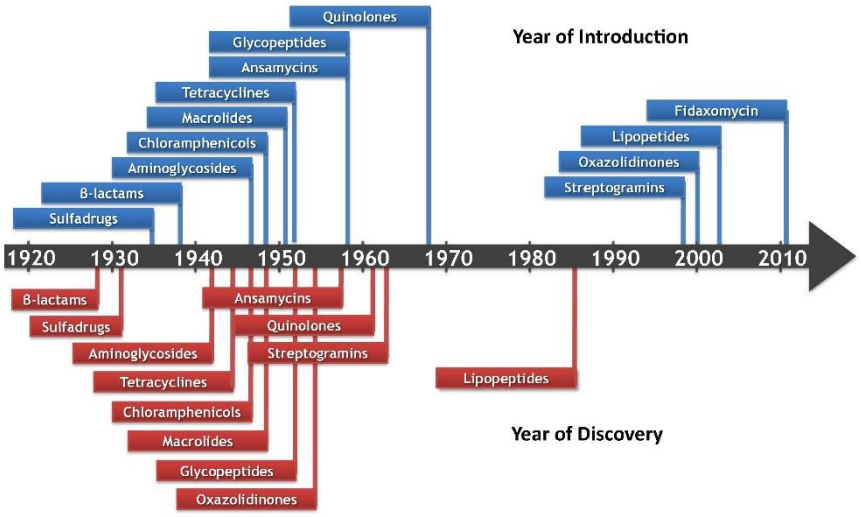

***Figure 1** - Timeline of antibiotic discovery and introduction into clinical practice (adapted from (Lewis, 2017)).*

Antibacterial agents can also be classified depending on the variety and diversity of susceptible bacterial species, being classified as narrow-spectrum, intermediate-spectrum and broad-spectrum antibiotics. Antibiotics with a narrow spectrum of action have limited applicability, as they are effective only on a small range of bacteria and may exhibit activity against Gram-positive or Gram-negative bacteria only. On the contrary, broad-spectrum antibiotics are those that are effective against a wide variety of bacteria, including Gram-positive and Gram-negative bacteria (Madigan *et al.*, 2016).

Antimicrobial consumption has been increasing over the years, but patterns and trends differ between countries. A study by Klein *et al.* (2018) on antibiotic consumption in 76 counties between 2000-2015 demonstrated that global antibiotic consumption increased 65%. This fact was primarily due to increased consumption in low- and middle-income countries (LMICs), since in 2000, high-income countries (HICs) had the highest antibiotic consumption rates, but in 2015, four of the six countries with the highest consumption rates were LMICs. In the most recent years, at the top of the list with the highest antibiotic consumption is Mongolia, Turkey, Greece and Iran (WHO, 2018).

Aside their therapeutic power, antibiotics are used for other purposes in the food and animal industry such as animal treatment, infection prevention and growth promoters (Davies and Behroozian, 2020).

Resistance to antibiotics occurs when bacteria multiply and adapt in their presence, decreasing the effectiveness of the drugs. Antibiotic consumption is a primary driver of antibiotic resistance and the wide and indiscriminately use of antibiotics enhanced the development of resistance. Over the past 50 years the availability of effective antibiotics for the treatment and prevention of infectious diseases has declined, once there is already resistance to almost all classes of antibiotics and only two new classes of antibiotics were commercialized (Waglechner and Wright, 2017; Sultan *et al.*, 2018; Davies and Behroozian, 2020).

2.1. Mechanisms of action

For the treatment of an infection, taking into account the difference between prokaryotic and eukaryotic cells in terms of structure and biosynthesis, the ideal would be to administer an antibiotic that only affects bacteria, without harming human and animal cells (Llarrull *et al.*, 2010).

As mentioned, besides being classified according to their structure and susceptibility, antibiotics can also be grouped according to their mechanism of action, the main ones being: inhibition of cell wall synthesis, inhibition of cell membrane synthesis, inhibition of protein synthesis, inhibition of nucleic acid synthesis and inhibition of metabolic pathways in bacteria (Reygaert, 2018).

Some antibiotics, like β-lactams, act by inhibition of cell wall synthesis. Antibiotics in this class lead to lysis, and consequent death of the bacterial cell. The peptidoglycan present in the bacterial cell wall gives it rigidity and protects the bacteria from osmotic lysis. Thus, the most effective process to prevent bacterial growth is to prevent cell wall synthesis, inhibiting the formation of the peptidoglycan network (Bush, 2012).

Polymyxins, for example, inhibit of cell membrane synthesis. The cell membrane acts as a selective barrier that controls the inner constitution of the cell, affecting its permeability. When these membrane functions are deregulated, ions and macromolecules present inside the cell are expelled, which results in cell death and lysis. The antibacterial agents show specificity for polysaccharides present on the outer membrane of many bacteria, mainly in Gram-negative bacteria. Since Gram-positive bacteria have an excessively thick cell wall, antibiotics of this type have a reduced efficacy in this type of bacteria (Llarrull *et al.*, 2010).

Protein synthesis is one of the most important functions in bacterial cells, thus, it is an important antibiotic target. Drugs that inhibit protein synthesis are part of the broader classes of antibiotics, which can be divided into two subclasses, 50S inhibitors (chloramphenicol, erythromycin) and 30S inhibitors (tetracycline, gentamicin, streptomycin). In both, these

inhibitors act by physically blocking the protein synthesis initiation process or interfering with the elongation process (Madigan *et al.*, 2016).

Other antibiotics inhibit the nucleic acid synthesis. Compounds of this class bind to components involved in the DNA and RNA synthesis process, compromising the multiplication and survival of the bacteria. RNA inhibitors interfere with the bacterial transcription process, in which the messenger RNA genetic information encodes protein synthesis. An example of this type of antibacterial agent is rifampicin. DNA inhibitors include some members of the quinolone class. Quinolones bind to DNA gyrases, inhibiting the function of these enzymes and interrupting DNA replication (Kohanski, Dwyer and Collins, 2010).

Antibacterial agents that inhibit the metabolic pathways generally act by inhibit the bacteria metabolism without interfering with the host metabolism, by only interfere with reactions catalyzed by enzymes that are only present in the bacterial cell. Two examples of this type of antibacterial agents are sulfonamides and trimethoprim, which interrupt folic acid synthesis, a necessary step in the production of important precursors of bacterial DNA and RNA synthesis (Kohanski, Dwyer and Collins, 2010).

Figure 2 lists in more detail the targets of the main antibacterial agents that are classified according to their target structures in the bacterial cell.

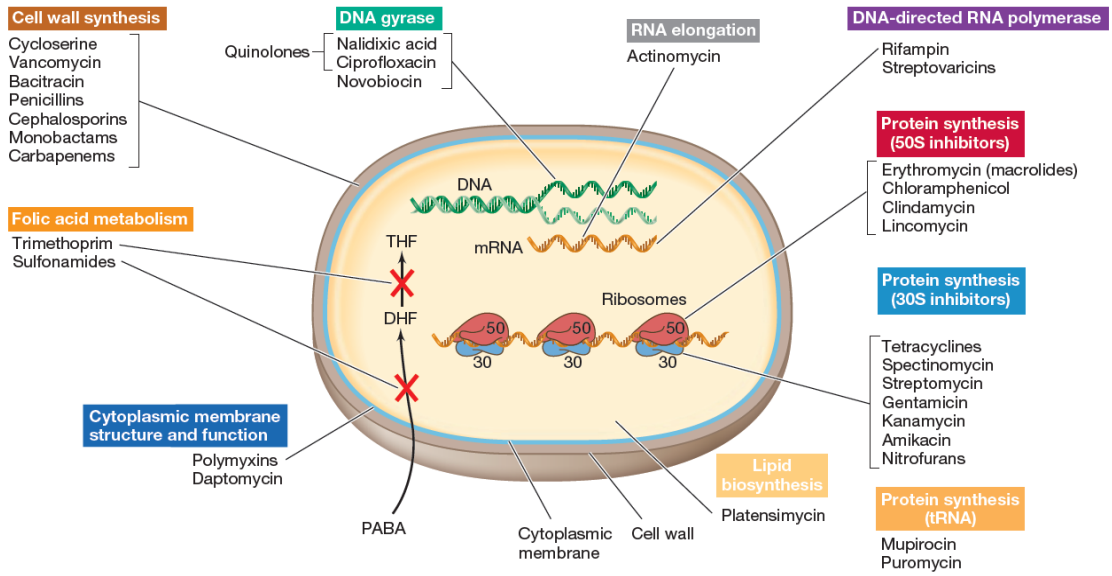

***Figure 2** - Targets of major antibacterial agentsad (adapted from (Madigan et al., 2016)).*

2.2. β-lactam antibiotics

Currently, β-lactam antibiotics constitute the most important group of antimicrobial agents in the hospital environment. They are the most frequently prescribed antibiotics given their

safety, therapeutic efficacy, low toxicity and broad spectrum of activity (Bush and Bradford, 2019).

β-lactams include penicillins, cephalosporins, carbapenems, monobactams and β-lactamase inhibitors. All these subclasses share the same molecular structure, formed by a β-lactam ring, and differ in the side chains (Figure 3).

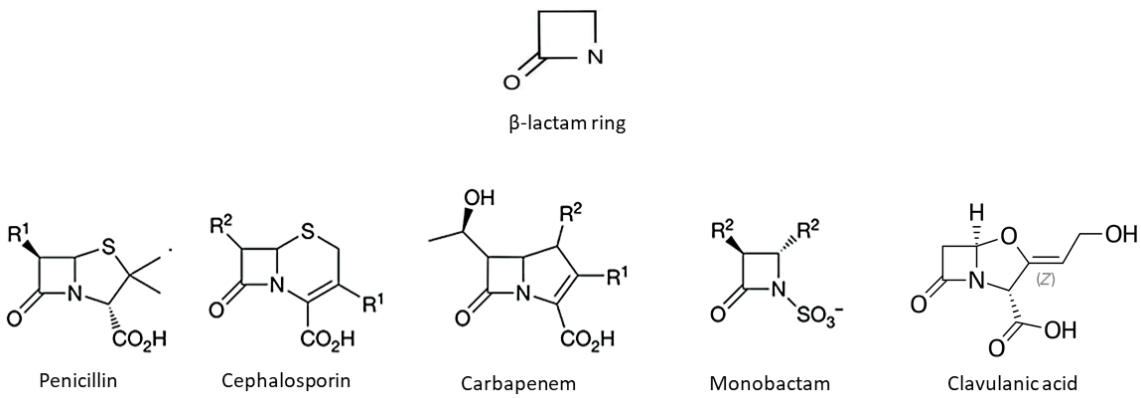

Figure 3 *- Representation of the chemical structure of β-lactam antibiotics, with emphasis on the β-lactam ring (adapted from (Bush and Bradford, 2019)).*

In 1941, penicillin was introduced as a therapeutic option. This completely changed the treatment of infectious diseases since, given its broad spectrum of activity, penicillin act against Gram-negative and Gram-positive bacteria (Bush and Bradford, 2016). However, it was in 1940 that a bacterial penicillinase was discovered. From that time onwards, penicillin became widely used, and thus resistant strains capable of inactivating the drug became prevalent. Synthetic studies were undertaken to modify penicillin chemically to prevent cleavage by penicillinases (β-lactamases) (Davies and Davies, 2010).

2.2.1. Mechanisms of action

β-lactam antibiotics are bactericidal agents that interrupt the formation of the cell wall as a result of covalent binding to penicillin-binding proteins (PBPs) (Bush and Bradford, 2019).

Peptidoglycan is a crucial constituent of the bacterial cell wall that provides mechanical stability to it. PBPs are enzymes that are necessary in the final steps of cell wall synthesis. The β-lactam antibiotics inhibit the last step in peptidoglycan synthesis by acylating the transpeptidase involved in cross-linking peptides to form peptidoglycan and the targets for the

actions of β-lactam antibiotics are the PBPs. This binding interrupts the terminal transpeptidation process and induces loss of viability and lysis. Each bacterial species has its own distinctive set of PBPs ranging from 3 to 8 enzymes per species (Bush and Bradford, 2016).

2.2.2. Carbapenems

Among β-lactam antibiotics, carbapenems have the greatest spectrum of action against Gram-negative and Gram-positive bacteria, and for that reason represent a last line in the fight against severe or high-risk bacterial infections and normally are reserved for multidrug-resistant (MDR) bacterial infections (Llarrull *et al.*, 2010; Codjoe and Donkor, 2017).

Carbapenems, such as imipenem, meropenem and ertapenem, are mostly used for treatment of UTIs, IAIs and lower respiratory tract infections (Codjoe and Donkor, 2017).

Carbapenems have a high penetration through the porin channels, still presenting resistance against the hydrolysis of most β-lactamases. However, carbapenemases, enzymes that recognize and hydrolyze almost all types of β-lactams, are a major problem associated with this powerful group. Even so, carbapenems are undoubtedly a strong weapon in fighting bacteria, and are the most active compounds against extended spectrum β-lactamases (ESBL) producing strains of the Enterobacteriaceae family (Papp-Wallace *et al.*, 2011; Kaye and Pogue, 2015).

3. Antibiotic resistance

Bacterial infections have been one of the main causes of disease throughout human history and apparently the appearance of antibiotics has solved this problem (Reygaert, 2018). According to the WHO, resistance to antibiotics is the reduction in effectiveness of a drug, and it occurs when bacteria multiply and adapt in their presence. The rapid evolution of microbial resistance is ancient, irreversible and inevitable and with the appearance of antibiotics, the appearance of antibiotic resistance increased (Dcosta *et al.*, 2011).

In recent years, antimicrobial resistance has rapidly emerged at a global scale and spread from one country to another faster than previously thought (Davies and Behroozian, 2020). Following resistance to penicillin discovered shortly after its clinical use, were discovered and introduced new antibiotics, such as tetracycline, chloramphenicol, and erythromycin, which were successful in treatment of penicillin resistant strains. Nevertheless, resistant strains to these antibiotics also started to appear. The warning of resistance was sounded nearly 70 years

ago with an enzyme with the ability to hydrolyze penicillin in *E. coli* and is now supported by the presence of resistant genes in nearly all environments (Miao, Davies and Davies, 2011; Pitout *et al.*, 2020).

The main factors contributing to the increase in this resistance are the widespread use, overuse and inadequate use of antimicrobial agents in the different areas such as clinical, agriculture, food, and improper prescription of antimicrobial therapy (Christaki, Marcou and Tofarides, 2020). The key to antibiotic resistance is antibiotic consumption, but the causes leading to increased antimicrobial resistance differ between countries. While in developed countries there is more indiscriminate prescription of antibiotics, in the developing world, the unregulated availability of antibiotics in community leads to reckless self-medication (Morehead and Scarbrough, 2018). Antibiotic resistance is more severe in Asia, southeast Europe, South America, and Africa (Bassetti *et al.*, 2017).

Currently, there is a wide range of antimicrobial agents so that we can choose which is the best to be used in the therapy of a potential infection, however resistance to all of them has already been reported, resistance that occurs right after an antimicrobial is approved for use (Figure 4) (Reygaert, 2018).

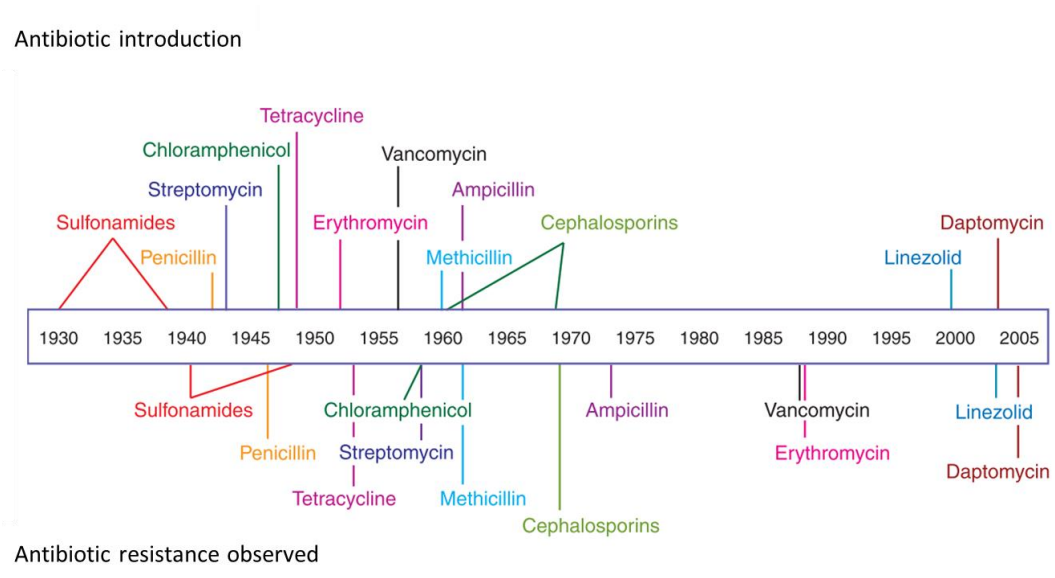

Figure 4 - *Timeline of antibiotic deployment and the evolution of antibiotic resistance (Adapted from (Clatworthy, Pierson and Hung, 2007)).*

As antibiotic use increased on a worldwide scale, it was discovered that antibiotic resistance developed not only by mutation, but also by an alternative mechanism: horizontal gene transfer (HGT). So, based on its origin, resistance can be classified as natural or acquired. Natural resistance can be divided into intrinsic resistance, which is always expressed in all members of

the species, which means that is universally shared by the species and is independent of antibiotic exposure (not related to HGT), or induced resistance, where genes are present naturally in the bacterium, but are only expressed after exposure to an antibiotic (Davies, 2014).

Regarding acquired resistance, acquisition of genetic material which confers resistance is possible by any route whereby bacteria acquires any genetic material: transformation, transposition, conjugation and besides, it can also be due to mutations in their own chromosomal DNA. This acquisition can be temporary or permanent (Kaye and Pogue, 2015).

A wide range of biochemical and physiological mechanisms may be responsible for both forms of resistance. Over the last 70 years many reviews have been describing the genetics and biochemistry of evolution and mechanisms of antibiotic resistance (Davies and Davies, 2010). Biochemical mechanisms of antibiotic resistance include increased efflux, enzymatic inactivation, target modification, target repair, protection, biofilm formation, decreased influx, sequestration, target bypass, target amplification and intracellular localization. The majority of these mechanisms are subject to HGT (Miao, Davies and Davies, 2011).

The mechanisms encoded by transmissible antibiotic resistance genes usually result in enzymatic inactivation of the antibiotics, either outside or inside the bacterial cell, for example, since the 1960s, hundreds of β-lactamases and other antibiotic inactivating enzymes have been characterized (Bush, 2010). Various databases list the existence of more than 20 000 potential resistance genes (r genes) but fortunately the number existing as functional resistance determinants in pathogens is much smaller (Davies and Davies, 2010).

However, there are many other mechanisms of resistance, many of novel function and not derived by mutation (Miao, Davies and Davies, 2011). A resistant strain may possess multiple mechanisms of resistance to the same antibiotic. Plasmid-mediated transfer of antibiotic resistance has been a major focus of investigation because of its medical and practical significance (Davies and Davies, 2010). Furthermore, expression of a resistance phenotype can be dependent on both the bacterial strain and the environment. What happens in laboratories does not necessarily reflect bacterial behaviour in the clinic: it is highly unlikely that clinical antibiotic resistance was based on a single mutation, as pathogens must have to overcome various forms of stress during the course of human infection (Miao, Davies and Davies, 2011).

A group of utmost concern are the Gram-negative bacteria are surrounded by the outer membrane, a permeability barrier for many substances including antibiotics. The low permeability of the bacterial outer membrane to specific antibiotic agents is responsible for the intrinsic resistance of some Gram-negative bacteria to those antibiotics. Moreover, changes in the outer membrane permeability can contribute to the development of acquired resistance. Porins are the major route of entry of hydrophilic antibiotics (such as β-lactams,

fluoroquinolones, tetracyclines, and chloramphenicol) through the bacterial outer membrane (Christaki, Marcou and Tofarides, 2020).

One of the most worrisome threats are the ESKAPE pathogen group (*Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* spp.) which are microorganisms responsible for increasing the number of resistance of most hospital-acquired infections because manage to "escape" the effects of the antimicrobial agents (Theuretzbacher, 2017).

The emergence of MDR bacteria worldwide has created a global crisis in medicine (Davies and Behroozian, 2020). Many of the bacterial pathogens associated with epidemics of human disease have evolved into MDR forms subsequent to antibiotic use (Davies and Davies, 2010).

3.1. Mechanisms of β-lactam resistance

There are two main resistance mechanisms for β-lactams, according to the microorganism in question: Gram-positive or Gram-negative bacteria.

In Gram-positive bacteria the most common mechanism is the acquisition of new PBPs that have less affinity for common β-lactams. In Gram-negative bacteria, the most common mechanism is the production of β-lactamases. But β-lactamases-producing bacteria are highly adaptive pathogenic microorganisms that also acquire resistance to antimicrobial agents through other mechanisms such as efflux pumps that expel β-lactams out of the cell, modification or deletion of the porin channels in specific β-lactams that reduce the entry of β-lactams. Loss of functionality of the porin channels can occur simultaneously with the production of β-lactamases, which leads to high levels of resistance (Llarrull *et al.*, 2010; Kaye and Pogue, 2015). Figure 5 shows a simple schematic representation of these mechanisms (Bush and Bradford, 2019).

The most prevalent Gram-negative pathogens, *E. coli*, *S. enterica*, *K. pneumoniae*, as already mentioned, can cause a variety of diseases in humans and animals, and a strong correlation between antibiotic use in the treatment of these diseases and antibiotic resistance development has been observed over the past half-century. This is especially apparent with the β-lactam class of antibiotics and their related inactivating enzymes, the β-lactamases. At this time, have been identified more than 2000 resistance-related β-lactamases (Pitout *et al.*, 2020).

HGT has played a predominant role in evolution and transmission of resistance to the β-lactam antibiotics among the enteric bacteria in both community and hospital infections (Davies and Davies, 2010).

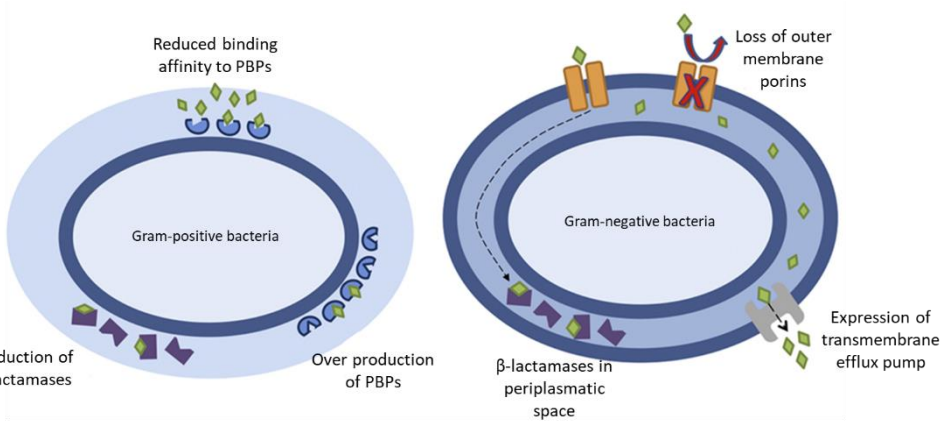

Figure 5 – *Representation of major mechanisms of β-lactam resistance in Gram-positive and Gram-negative bacteria (adapted from (Tang, Apisarnthanarak and Hsu, 2014)).*

3.2. β-lactamases

β-lactamases are bacterial enzymes that inactivate β-lactam antibiotics by hydrolysis of the β-lactam ring, which results in ineffective compounds and, as stated earlier, are the most common mechanism of bacterial resistance by Gram-negative bacteria to β-lactam antibiotics (Bonomo, 2017; Khan, Miller and Arias, 2018; Christaki, Marcou and Tofarides, 2020).

The first β-lactamases were described in 1940, one year before the introduction of penicillin in clinical practice (Christaki, Marcou and Tofarides, 2020). More recently, an example of fast spread since first being reported in 2010 is the New Delhi metallo-β-lactamase-resistance gene (*bla*$_{\text{NDM-1}}$), which confers resistance to penicillin, cephalosporins and a range of their derivatives. *bla*$_{\text{NDM-1}}$ is associated with other resistance determinants and has resulted in increasing mortality (Bush *et al.*, 2011).

3.2.1. β-lactamases classification

Currently are used two main β-lactamases classifications systems: the Ambler molecular classification system, which separates β-lactamases in classes A to D, based on amino acid homology and the Bush-Jacoby-Medeiros functional system that classifies enzymes in 4 fundamental groups, from 1 to 3, with several subgroups within 2, based on enzymatic studies to determine relative hydrolysis rates of a varied set of β-lactam substrates and inhibition profiles by β-lactamase inhibitors (Bush and Bradford, 2019). Table 1 shows a summary of these two classification systems.

Table 1 *- Classification of β-lactamases based on Amber and Bush-Jacoby-Medeiros system (adapted from (Bush and Jacoby, 2010; Bush, 2018; Ur Rahman et al., 2018)).*

Active Site	Molecular class	Functional group	Major functional subgroup	Known substrates	Representative enzymes
Serine	A	2	2a	Penicillins	PC-1
			2b	Penicillin, cephalosporin (some 1st-generation)	TEM-1, TEM-2, SHV-1
			2be	Penicillin, Cephalosporin, Expanded-spectrum cephalosporin, Monobactam	CTX-M (CTX-M-15), ESBLs (TEM-3, SHV-2), PET-1, VEB-2
			2br	Penicillin	IRT, TEM-30, SHV-10
			2ber	Extended spectrum cephalosporin, monobactam	TEM-50
			2c	Penicillin (Carbenicillin)	CARB-1, CARB-3, PSE-1
			2ce	Extended spectrum β-lactams	CepA
			2f	Penicillin, Cephalosporin, Carbapenem, Expanded-spectrum cephalosporin, Monobactam	KPC-2, SME-1, IMI-1
	C	1	1	Cephalosporins	AmpC, CMY-2, P99, ACT-1, FOX-1, MIR-1
			1e	Cephalosporins, Expanded-spectrum cephalosporin	GC1, CMY-37
	D	2d	2d	Penicillin	OXA-1, OXA-10
			2de	Penicillin, Expanded-spectrum cephalosporin, Monobactam	OXA-11, OXA-15
			2df	Penicillin, Carbapenem	OXA-23, OXA-48
Metallo (Zinc)	B	3	3a	Penicillin, Cephalosporin, Expanded-spectrum cephalosporin, Carbapenems	IMP-1, VIM-1, NDM-1, CcrA, IND-1
			3b	Carbapenems	CphA, Sfh-1

Within the Ambler system, β-lactamases are distinguished according to their active enzyme center, classes A, C and D are serine β-lactamases and class B is metallo-β-lactamase (MBL). Class A, also known as penicillinases, is the class that has the largest number of enzymes. Carbapenemases are the most versatile family of β-lactamases, presenting a spectrum of action that is capable of hydrolyze almost all β-lactam antibiotics. (Bush, Jacoby and Medeiros, 1995). Classes A to C have been well documented as both chromosomally encoded and plasmid mediated enzymes. The class D β-lactamases have been much more elusive and, for the most part, were identifiable only as plasmid-encoded β-lactamases in Gram-negative bacteria (Evans and Amyes, 2014).

The introduction of third-generation cephalosporins in the early 1980s was quickly followed by the identification of plasmid-encoded β-lactamases capable of hydrolyzing third-generation cephalosporins (ESBLs) in 1983 (Christaki, Marcou and Tofarides, 2020).

3.2.2. Action spectrum and dissemination

Genes encoding β-lactamases can be found in the chromosome or in mobile genetic elements (MGEs), which facilitates their dissemination among bacteria (Christaki, Marcou and Tofarides, 2020). Gene transfer and gene expression within phylogenetically related bacteria occur frequently in nature (Miao, Davies and Davies, 2011).

Carbapenemase-producing Enterobacteriaceae (CPE) have been found worldwide. Horizontal transmission of carbapenemase genes, mediated by MGEs that carry additional resistance elements, which confer resistance to various groups of antibiotics, results in resistance to multiple drugs, including bacteria resistant to all available antibiotics (Hrabák, Chudáčková and Papagiannitsis, 2014). Carbapenemase-producing microorganisms spread clonally from individual to individual and genes encoding carbapenemase can spread evenly between colonies (Nasser, 2017).

Gene transfer between bacteria is considered to be ancient and universal and is an important element in theories of cellular evolution and in the formation and maintenance of microbial communities. Putative antibiotic resistance genes have been detected in isolated human populations never exposed to antibiotics, and similar gene families are present in microbial communities from all sources that have been examined including human, animal, plant microbiomes and even ancient environments (Davies, 2014). Shoemaker et al. (2001) demonstrated extensive HGT in the gut, indicating that the human gastrointestinal tract is a major source of antibiotic resistance genes (Miao, Davies and Davies, 2011).

β-lactamases are chromosomally encoded in many environmental bacteria but are more frequently encoded, in many variations, on plasmids in pathogens. It has been identified a strong relationship in CTX-M β-lactamase genes that originated from a soil *Kluyvera* spp. bacterium. The huge β-lactamase family of enzymes is clearly of diverse origins. In addition, the continuing *in situ* evolution by mutation concurrent with the use of chemically synthesized derivatives of β-lactam antibiotics ensure their omnipresence (Miao, Davies and Davies, 2011). Although the rapid dissemination of New Delhi MBL (NDM) producing Enterobacteriaceae resembles that of *Klebsiella pneumoniae* carbapenemase (KPC) producing Enterobacteriaceae, the spread of NDM-type MBLs does not appear to be associated with dominate clonal strains and is mediated by several different plasmid incompatibility (Inc) types (Logan and Weinstein, 2017).

3.2.3. Carbapenemases

Phenotypic resistance to carbapenems is typically caused by two main mechanisms: β-lactamase activity combined with structural mutations or production of carbapenemases. Carbapenemases are enzymes that hydrolyze carbapenem antibiotics. They are classified by their molecular structures and belong to 3 classes of β-lactamases: class A, B, and D of the Ambler classification system (Logan and Weinstein, 2017).

As carbapenem-resistant Enterobacteriaceae (CRE) have become increasingly prevalent worldwide, carbapenems, a last line of defense, are increasingly being challenged by MGEs harboring carbapenemases and other drug resistance genes (Logan and Weinstein, 2017). A lack of alternative treatment options has led to a mortality rate of up to 50% for infections with carbapenem-resistant strains (Kelly, Mathema and Larson, 2017). CRE, most notably *E. coli* and *K. pneumoniae*, which were relatively uncommon before 2000, have doubled in prevalence over the following decade among HAICs and currently are also prevalent in the community (Martínez-Martínez and González-López, 2014).

Increasing numbers of Enterobacteriaceae are reported as frequent carriers of genes encoding two of the most concerning subclasses of carbapenemases: KPC, which has become endemic in parts of the America, southern Europe, Israel and China; and NDM, which has become endemic in northern Europe and the Asia Pacific region (Bush, 2013). Besides NDM, two other forms of metallo-β-lactamase carbapenemases, namely Verona integron-encoded metallo-β-lactamase (VIM) and imipenemase metallo-β-lactamase (IMP), are less common but are equally concerning. ESBLs and ampicillin hydrolyzing enzymes (AmpCs) are also capable of conferring carbapenem resistance when combined with the mutation of porins in Gram-negative bacteria (Logan and Weinstein, 2017).

3.2.4. Oxacillinases

The OXA β-lactamases were the first β-lactamases to be found and initially these enzymes of molecular class D were relatively rare and always mediated by plasmids, had a substrate limited to penicillins and some to cephalosporins. It was quickly discovered that all strains of *A. baumannii* have an OXA β-lactamase chromosomally encoded, some of which confer resistance to carbapenems. In some cases, carbapenem-resistant OXA β-lactamases have migrated to Enterobacteriaceae and are becoming a significant cause of carbapenem resistance (Evans and Amyes, 2014).

OXA-48 is the main carbapenem-hydrolyzing class D β-lactamase found in Enterobacteriaceae and was initially identified in 2001 and was obtained from a *Klebsiella pneumoniae* isolate from a patient in Istanbul, Turkey. OXA-48 represents one of the most worrying developments in carbapenem resistance in the last decade (Nasser, 2017).

OXA-48-type oxacillinase is the third most prevalent carbapenemase globally, found most often in North Africa and Europe. The most important OXA-48 *bla* gene was initially identified in *K. pneumoniae,* however, currently several types of Enterobacteriaceae that contain this gene are already known, for example, *Proteus mirabilis*, *Klebsiella oxytoca*, *Serratia marcescens*, *E. coli*, *Citrobacter freundii*, *Morganella morganii* and *Enterobacter* spp. and have also been reported in *A. baumannii.* β-lactamase OXA-48 hydrolyzes penicillins effectively but carbapenems weakly, and has little activity against extended-spectrum cephalosporins (Evans and Amyes, 2014; Dabos *et al.*, 2018)**.**

Scope

The general objective of this work is to study resistance to antibiotics, especially resistance to β-lactams, namely carbapenems, which has been increasing, particularly in hospital environment, as well as the problems that arise with that. Thus, this dissertation is divided into 3 chapters:

In chapter 1, the specific objective is to infer the epidemiology of β-lactamases producing strains in the CHBV, with an emphasis on the carbapenemase-producing strains, and try to understand which are the most common resistance mechanisms, studying the highest prevalence of resistant strains according to gender, age and hospital service patients, comparing results of nosocomial and community bacteria.

Chapter 2 aims to implement a protocol for manual extraction of nucleic acids to allow further molecular studies of carbapenemase-producing strains, for possible subsequent introduction into the CHBV laboratory routine.

Chapter 3 is a chapter of science communication and aims the development of pedagogical materials to raise awareness about antibiotic resistance.

The chapters are independent from each other.

Material and Methods

1. Central Hospital characterization

The present study was performed in the Microbiology laboratory of the Clinical Pathology department of the "Hospital Infante D. Pedro" (HIP) which is one of the three units that compose the "Centro Hospitalar do Baixo Vouga, E.P.E." (CHBV). CHVB provides differentiated health care to the various municipalities in the Aveiro district. HIP has several services such as internal medicine, general surgery, orthopedics, pediatrics, urology, infectiology, cardiology, pneumology, gynecology and obstetrics, intensive care medicine, neurology, psychiatry, and others.

The Clinical Pathology service is responsible for carrying out the laboratory tests necessary for laboratory diagnosis and monitoring of emergency room patients, inpatients and outpatients. It includes all the main functional areas of clinical and laboratory research such as clinical biochemistry, immunology and allergology, hematology and microbiology. In addition to the services provided to the hospital itself, it also provides laboratory support to other health institutions in the Aveiro region.

The main biological products analyzed in the microbiology laboratory are urine, pus, sputum and blood samples and the tests performed in the laboratory aim to diagnose, treat, monitor and prevent diseases, and the susceptibility tests performed to antimicrobials are essential for the success of patients treatment.

2. Samples

The isolates analyzed in this study were recovered from both inpatients and outpatients from different wards of the CHBV. From April 2019 to February 2020, β-lactam resistant strains were isolated from different samples (urine, civet and bladder puncture, blood, pus, sputum and lower respiratory tract samples) responsible for infection or colonization.

2.1. Laboratory routine

Samples from all the different wards of the CHBV are sent to the clinical pathology service to be analyzed. All samples are identified with the patient's clinical data which includes the type of sample, date of hospital admission, age, sex, hospital ward, type of infection, previous infections, subsequent therapies, and clinical results. Therefore, depending on the desired objective and the different types of biological products to be analyzed, the samples follow the established laboratory procedures.

Regarding the microbiology laboratory, the samples are inoculated in the appropriate culture medium, and incubated at 35ºC for different periods of time, depending on the collection site. Urine samples are inoculated in CLED (Cystein Lactose Electrolyte Deficient medium) (BioMérieux, France). 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 <103 CFU/ml, 10-100 CFU/ml and ≥105 CFU/ml, respectively. Sputum, pus and blood samples are inoculated either in MacConkey Agar (BioMérieux, France), a selective and differential medium, or Columbia Agar (BioMérieux, France), with 5% sheep blood or PolyViteX Chocolate Agar (BioMérieux, France), for later identification and antimicrobial susceptibility testing.

2.2. Identification of bacterial strains

The isolates included in this study were identified with the automated broth microdilution method VITEK®2 (BioMèrieux, France), in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (version 9.0, 2019). 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.

The identification was made from a fresh and pure culture, from where a suspension of 0.55 to 0.65 McFarland of bacteria is inoculated through a vacuum system into the chosen cards. The cards were then incubated at 35,5±1ºC and read every 15 minutes.

The results of 64 tests were taken into account, each containing an individual substrate, evaluating the metabolic activity of the organism: acidification, alcalinization, enzymatic hydrolysis or growth in the presence of inhibitors. 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) Gram-positive spore-former cocci. All cards contain a control well, where there is only culture medium. Each card contains a bar code, reporting the type of ID card, lot number, expiring date and the corresponding sample identification for the equipment.

The results are compared with a database of well characterized strains, and an ID is obtained with a certain degree of similarity of metabolic test. The identification usually requires from 18h to 24h of incubation in the VITEK®2 Reader/Incubator.

2.3. Antimicrobial Susceptibility Testing

After identification, the antimicrobial susceptibility test (AST) is performed 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). AST was also performed with VITEK®2, in accordance with the EUCAST guidelines (version 9.0, 2019).

In order to perform antimicrobial susceptibility testing, the inoculum previously performed for the identification is used. The pure and fresh culture is taken to a suspension of 0.55 to 0.65 McFarland. After that, this suspension is inoculated, through a vacuum system, into the chosen card. The results cards are then incubated at 35,5±1 ºC and read after 24 hours, and by the end are compared with a database of well-characterized strains, obtaining an ID with a certain degree of similarity of the metabolic test.

Each card has 64 microwells with selected antibiotics in different concentrations. The system monitors each of the wells for growth 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, the results obtained are expressed in sensitive, intermediate, or resistant phenotype, to a specific antibiotic with a MIC value, according to EUCAST guidelines (Version 9.0, 2019).

2.4. Phenotypic methods

2.4.1. RAPIDEC® CARBA NP

The RAPIDEC® CARBA NP (BioMèrieux, France) is a rapid biochemical test that identifies carbapenemase-producing bacteria by detecting the hydrolysis of the β-lactam ring of a carbapenem. It is a valuable test in clinical field due to its sensitivity and specificity of 96% and results in less than 2 hours sample preparation (Poirel and Nordmann, 2015).

This test detects carbapenem hydrolysis by carbapenemase-producing bacteria: *Enterobacteriaceae*, *Pseudomonas aeruginosa* e *Acinetobacter baumannii*. Hydrolysis acidifies the medium, resulting in the colour change of the pH indicator. After bacterial lysis that allows the extraction of the enzyme, the lysate is added to a detection solution containing imipenem, phenol red (pH indicator) and zinc, necessary for metal-dependent carbapenemase-producing strains. The RAPIDEC® CARBA NP kit detects, without distinction, 3 types of carbapenemases: KPC, metallo-β-lactamases (NDM-1, VIM and IMP) and OXA-type carbapenemases.

Results are obtained by the comparison of the colours of the control well and the test well. The results are positive when there is colour variation between the two wells, as shown in figure 6.

Figure 6 *– Positive results of CARBA NP test.*

2.4.2. CORIS *BioConcept* RESIST-3 O.K.N. K-SeT

CORIS *BioConcept* RESIST-3 O.K.N. K-SeT is a rapid immunochromatographic assay for the detection of OXA-48, KPC and NDM carbapenemases in a bacterial colony. A single colony of each isolate was suspended in 10 drops of lysis buffer. Then, three drops of the suspension were added onto the test strip. The results were read with the naked eye within 15 minutes at room temperature.

The results are read by comparing the control band and the OXA-48, KPC and NDM bands. Positive results show the band in the control position and the band in the present carbapenemase position, as it shows in figure 7.

Figure 7 *– Positive results of CORIS test. Presence of KPC and OXA carbapenemase, respectively.*

2.4.3. MICRONAUT-S broth microdilution Colistin MIC test

MICRONAUT-S broth microdilution Colistin MIC test (Merlin Diagnostika) is based on phenotypic detection of resistance as expressed by microbial growth in presence of colistin. This micro dilution procedure is a standardized method and a reference system for determination of the minimal inhibitory concentration.

A fresh and pure culture was used to achieve 0,5 McFarland standard turbidity suspension in a NaCl solution (DENSIMAT, BioMérieux). 50 µL of that solution were inoculated in 11,5 mL of Mueller-Hinton broth. Then, 100 µL of the obtained suspension was inoculated into each well of the plate-test, which contains a control well and an increasing concentration of colistin in the remaining wells. Finally, the plate must be sealed with the unperforated plate sealer and incubated at 35-37ºC for 18-24 hours. The results are interpreted visually, according to the manufactures instructions, being the MIC value reported as that where bacterial growth did not occur.

Chapter I. Epidemiology of carbapenem resistant strains

1. Introduction

Antibiotics are necessary for the treatment of bacterial infections. Currently, there are many different antimicrobial agents available, but β-lactams are the major antibiotic class used in the treatment of those infections. However, antibiotic resistance is a current major health concern as antimicrobial resistance in different pathogens have been increasing. Microorganisms have several mechanisms that allow them to adapt to stress and lead to resistance to several antimicrobials used in clinical practice, and one of those mechanisms is β-lactamase production (Khan et al. 2018; Mohamed et al. 2018).

β-lactamase production is most frequently suspected in a Gram-negative bacterial isolates that demonstrates resistance to a β-lactam antibiotic (Bush and Jacoby, 2010). Therefore, an early detection of these strains is necessary (Evans and Amyes, 2014). However, it is not always easy to obtain a rapid identification of these strains relaying only in phenotypic tests since the resistance determinants may sometimes confer only a slight increase of MIC values for carbapenems (Evans and Amyes, 2014).

β-lactamases groups of utmost clinical importance in Gram-negative bacteria are ESBLs, enzymes conferring resistance to penicillins, first-, second-, third-generation cephalosporins, and aztreonam but not cephamycins or carbapenems and which are inhibited by β-lactamase inhibitors; AmpC enzymes, conferring resistance to penicillins, first-, second-, third-generation cephalosporins, aztreonam, and cephamycins but not carbapenems and which are inhibited by β-lactamase inhibitors.

Within β-lactam antibiotics, carbapenems are considered one of the most reliable drugs for treating bacterial infections due to its broad spectrum antibacterial activity. Carbapenems are often the antimicrobials of last resort to treat infections associated with ESBLs, MBLs, or plasmid-mediated AmpCs (Codjoe and Donkor, 2017). One of the mechanisms of resistance to carbapenems is the production of carbapenemases by bacteria. Carbapenemases are a diverse group of enzymes conferring carbapenem resistance, many of which confer resistance to almost all hydrolysable β-lactams (Christaki, Marcou and Tofarides, 2020).

In Enterobacteriaceae, carbapenemases are the most important mechanism of carbapenems resistance. Carbapenemase-producing Enterobacteriaceae (CPE) have been found worldwide and carbapenemases like KPC, NDM and OXA demand special attention (Nasser, 2017).

KPC-type enzyme has been extensively reported in *K. pneumoniae* and is the most common enzyme disseminated among Enterobacteriaceae, since often are carried on a mobile plasmid. To date, among the several KPC variants that have been described, the bla_{KPC-2} and bla_{KPC-3} genes

account for the most bla_{KPC} (Van Duin *et al.*, 2014). In addition, a few studies reported the carbapenemase GES-5, a point mutant derivative of the ESBL GES-1 in *K. pneumoniae* (Navon-Venezia, Kondratyeva and Carattoli, 2017). In 2017, according to the annual report of the European Centre for Disease Prevention and Control (ECDC) on antimicrobial resistance in Europe, 8.6% of *K. pneumoniae* causing invasive infections in Portugal were resistant to carbapenems (Aires-De-Sousa *et al.*, 2019).

Metallo-β- lactamases, mainly NDM, VIM, and IMP-type enzymes, and OXA-48–like β-lactamases have also become widespread globally and are an urgent public health threat (Grundmann *et al.*, 2017; Khan, Miller and Arias, 2018).

OXA-48-like carbapenemases in isolation induce a relatively weak hydrolysis of penicillins and carbapenems but not cephalosporins. As a consequence they may be more difficult to detect. High level carbapenem resistance may occur when these enzymes are found in combination with other β-lactamases such as ESBL, or with porin changes leading to permeability defects (Logan and Weinstein, 2017; van Duin and Doi, 2017).

CPE isolates are usually resistant to many other β-lactam and non-β-lactam antibiotics, leading to multi-resistant isolates. According to a survey on the epidemiological situation for CPE in European countries, between 2010 and 2018, by Brolund *et al.* (2019), CPE cases tend to maintain or increasing, and comparing 2010 to 2018, countries like Greece, Italy, Malta and Turkey reported an endemic situation in 2018. In the timeframe of the study, Portugal had an increase in the epidemiological stage regarding spread of CPE, going from sporadic occurrences (epidemiologically unrelated single cases) in 2010, to regional spread (more than one epidemiologically related hospital outbreak confined to hospitals that are part of the same region or health district, indicating regional autochthonous inter-institutional transmission) in 2018 (Manageiro *et al.*, 2018).

2. Results and discussion

2.1. Characterization of collected samples

In this study, 52 carbapenems-resistant isolates from 48 patients of the CHBV were collected from 2019 (N=48) to 2020 (N=4). The number of isolates collected from men (n=28) is higher than number of strains collected from women (n=20) (figure 8).

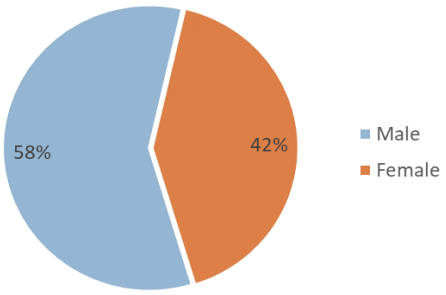

Figure 8 – *Patients distribution by gender.*

With exception of a 17 years old patient, patients ages ranged between 50 and 100 years old, with the majority being above 65 and the average being 73 years (figure 9). According to the Consortium on Resistance Against Carbapenems in *Klebsiella* and other Enterobacteriaceae (CRACKLE) (Van Duin *et al.*, 2014) the population with carbapenem-resistant organisms (CPOs) are mostly elderly patients, being the average age 70 years old. The elderly population is more vulnerable to bacterial infections, as increasing age, length of hospital stay, insertion of various types of catheters, the performance of certain types of invasive or surgical procedures and mechanical ventilation are risk factors for acquiring infections and, in this case, CPE infections (Mariappan, Sekar and Kamalanathan, 2017).

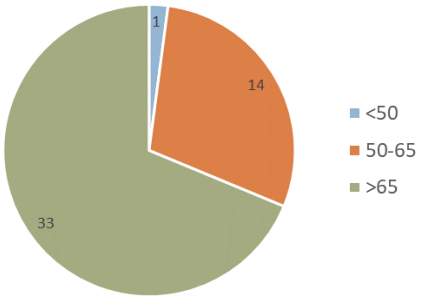

Figure 9 – *Patients distribution by age group.*

As to the provenance of patients, the samples were collected from patients from different wards of the CHBV. The samples were considered nosocomial when obtained from patients hospitalized in the different wards of the hospital, in this case, inpatients (n=28) attending Surgery, Medicine (Águeda), Medicine (Estarreja), Medicine I, Medicine II, Medicine III, Intensive care units, Neurology, Orthopedics, and Pneumology. The remaining samples were considered non-nosocomial once they were collected from outpatients (n=20) (patients attending emergency room (n=15), emergency room surgery (n=3), and emergency room pediatrics (n=1)) (table 2).

Table 2 - *Patients distribution by hospital ward.*

Hospital ward	SUR	MED Águeda	MED Estarreja	MED I	MED II	MED III	ICU	NEU	ORT	PNEUM	ER
Nº patients	4	2	4	3	6	5	1	1	1	1	20

SUR: Surgery; MED: Medicine; ICU: Intensive Care Units; NEU: Neurology; ORT: Orthopedics; PNEUM: Pneumology; ER: Emergency room.

It was assumed that samples collected from outpatients represent the community. Thus, a higher number of carbapenem resistant strains was observed in inpatients (figure 10), which shows that CPOs are a serious threat in the healthcare facilities and have been disseminated further since 2015. However, the number of carbapenemase-producing isolates collected from outpatients was surprisingly high. This fact reflects the spread of these CPE through the community (Brolund *et al.*, 2019).

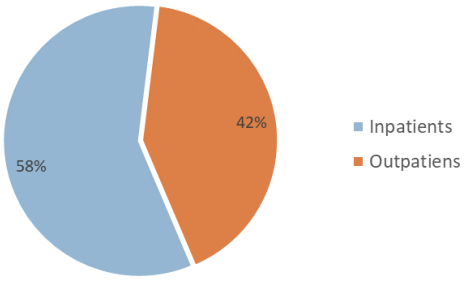

Figure 10 – *Distribution of carbapenem-resistant strains considering whether they are from inpatients or outpatients.*

Regarding the biological products, carbapenems-resistant isolates collected were isolated mainly from urine (n=35), followed by blood (n=6), pus (n=3), sputum (n=3), endotracheal suction (n=1), bronchial aspirate (n=1) and bronchoalveolar lavage (n=1) samples. Urine stands out as the main biological product of reference for microbiological laboratory analysis since,

urinary tract infections are the most common and most frequently observed pathologies, thus justifying its predominance over other harvested biological products.

2.2. Characterization of carbapenem-resistant isolates collected

In a total of 51 samples collected, 52 isolates were identified, since it was possible to isolate two strains from the same pus sample of an inpatient attending the surgery ward. All carbapenem-resistant isolates identified were Enterobacteriaceae: *K. pneumoniae*, *E. coli*, *E. cloacae* and *S. marcescens* (figure 11). Carbapenem-resistant Enterobacteriaceae (CRE) are common infectious agents in hospital care, and are becoming more prevalente in the community (Logan and Weinstein, 2017).

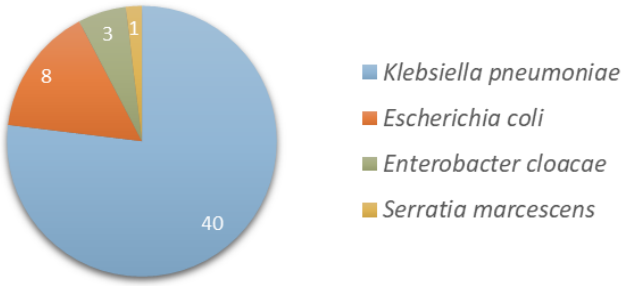

Figure 11 – *Number of carbapenem-resistant strains identified.*

K. pneumoniae was the most prevalent microorganisms found, followed by *E. coli*, both in urine samples. Figure 12 shows the relationship between the identified microorganisms and the biological samples where they were collected.

Although it was not possible to confirm whether the microorganisms present in the urine samples were causing infection or colonization, it is likely that these cases are associated with urinary tract infections (UTIs) once *K. pneumoniae* and *E. coli* are two important opportunistic pathogens in the hospital environment and the community and are very commom causes of UTIs (Martin and Bachman, 2018).

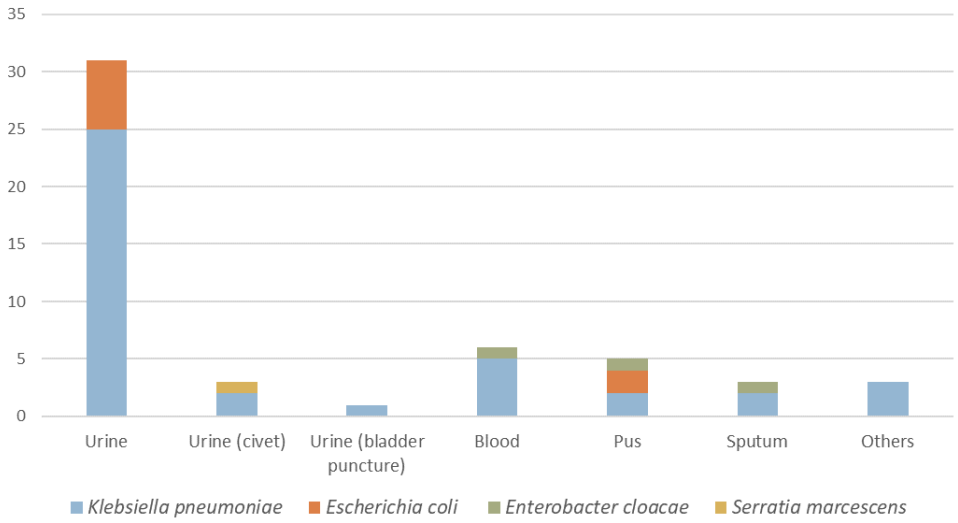

Figure 12 – *Bacterial strains distributed by samples.*

In addition to the susceptibility profile resistant to β-lactams demonstrated by all isolates, all samples analyzed showed sensitivity to colistin (values between <0.5 and 2 µg/mL), with the exception of *E. cloacae*, that showed resistance to colistin (16 µg/mL), which is not surprising since this species shows intrinsic resistance to colistin.

Analyzing the strains, it is possible to conclude about the β-lactam-resistant mechanisms present. 51 β-lactamases enzymes were detected, of which 45 carbapenemases (43 *Klebsiella pneumonia*e carbapenemase (KPC) and 2 OXA-48), 5 AmpCs and 1 ESBL (figure 13).

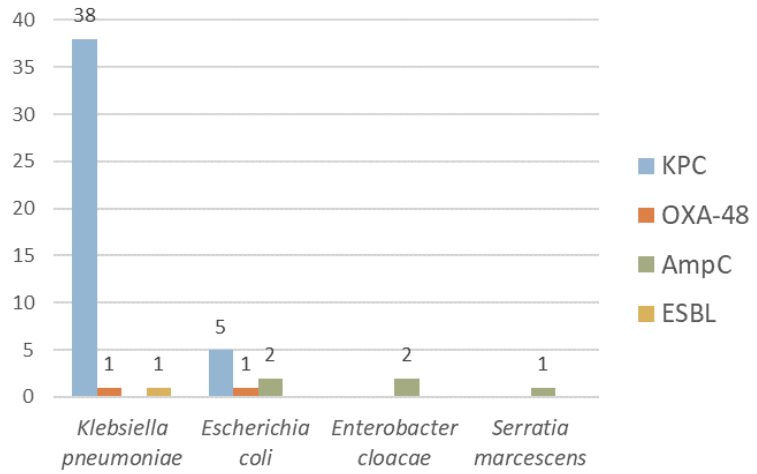

Figure 13 – *β-lactamases present in the bacterial strains.*

Of the 43 KPC identified, the majority was found in *K. pneumoniae*, followed by *E. coli.*

One *E. cloacae* isolate was identified as resistant to carbapenems, but according to the tests described in Material and Methods section, no enzyme was identified, which indicates that another mechanism of resistance to carbapenems must be present.

Resistance to carbapenems in non-carbapenemase producers can be be explained by other resistance mechanisms such as lack of porins or mutations capable of modifying the structure of porin proteins and, thus, reducing the influx, and efflux pumps capable of developing an active transport mechanism that pumps antibiotic molecules that penetrate the cell outwards (Kaye and Pogue, 2015).

When comparing the β-lactamases identified, and in accordance with the fact that there was a higher incidence of inpatients, it is possible to conclude that there is a higher prevalence of these enzymes in nosocomial microorganisms than non-nosocomial ones (figure 14), although the difference is not very accentuated.

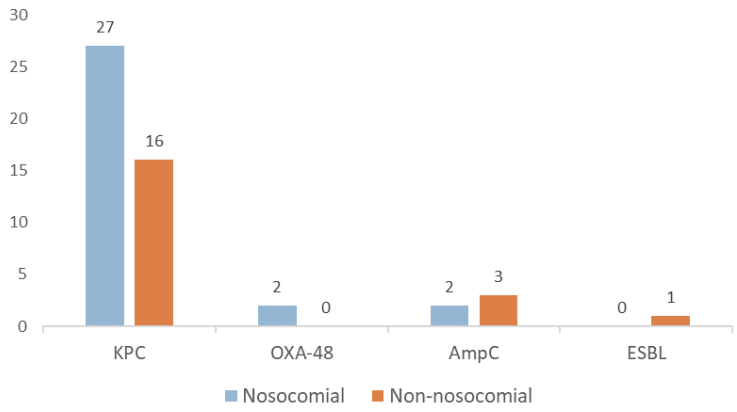

Figure 14 – *Distribution of the β-lactamases detected considering if they were nosocomial or non-nosocomial.*

2.2.1. *Klebsiella pneumoniae* carbapenemases (KPC)

As mentioned, of the 51 β-lactamases enzymes detected in the CRE isolates, 43 were KPC enzymes. Carbapenemases of the KPC family have the most extensive global distribution of all carbapenemases associated with Enterobacteriaceae and the most common species of Enterobacteriaceae harboring transmissible carbapenemase genes are *K. pneumoniae* (Logan and Weinstein, 2017; van Duin and Doi, 2017).

The KPC enzimes and their dissemination poses a major problem. In one hand due to their capability of conferring resistance to many antibiotics, on the other hand, these enzymes usually

are located in MGEs such as plasmids, which enhances their dissemination. KPC enzymes have disseminated rapidly throughout the globe and have been detected in virtually all clinically relevant Enterobacteriaceae (Munoz-Price *et al.*, 2013). The KPC enzyme is usually encoded by the bla_{KPC} gene. Plasmids with bla_{KPC} undergo horizontal transfer through conjugation with other bacterial cells. Thus, rapid movement of bla_{KPC} from cell-to-cell is a major contributor to its general spread and accompanying resistance determinants (Porreca, Sullivan and Gallagher, 2018). Also, these plasmids carry other resistances genes that all togheter with the KPC enzyme turns the microorganisms that carries it a superbug. Generally, KPC-producing organisms tend to be multidrug resistant once bla_{KPC} is carried on large plasmids with accompanying resistance determinants, including those responsible for resistance to aminoglycosides, quinolones, trimethoprim, sulfonamides, and tetracyclines (Deshpande *et al.*, 2006).

Several KPC variants have been reported worldwide, with KPC-2 and KPC-3 being the most prevalent (Stoesser *et al.*, 2017). In Portugal, the spread of carbapenemase genes have been verified, as well as the widespread distribution of bla_{KPC-3} among *K. pneumoniae* isolates in different hospitals (Rodrigues *et al.*, 2016; Tacão *et al.*, 2017). Although it was not possible to perform the detection of genes in this study, the presence of bla_{KPC} genes have been reported in the CHBV, with bla_{KPC-2} and bla_{KPC-3} genes accounted for most of bla_{KPC} (Silva, 2019).

Among the KPC enzymes found in this study, the majority were nosocomial (figure 14). Currently, KPC-producing organisms have been described primarily in healthcare settings. Risk factors for acquisition of KPC-producing organisms are not specific, but include exposure to acute care hospitals or long-term care facilities, transfer of patients between hospitals with high KPC levels, prior antibiotic use, elevated colonization pressure in endemic settings, and the prolonged use of indwelling and central venous catheters (Van Duin *et al.*, 2014).

Infections due to *K. pneumoniae* with carbapenemases often reach mortality rates ranging between 23% and 75%, which are attributed to the lack of active antimicrobial agents and underlying comorbidities of patients (Karaiskos and Giamarellou, 2014).

From the infection control point of view, dealing with patients infected or colonized by bacteria carrying these enzymes poses a major challenge. Several isolation measures have to be taken which implies more costs to the hospital. From the patient point of view these measures can be devastating since sometimes they imply that the patient can not receive visits. Source control, in addition to antimicrobial therapy, is essential for the effective management of these infections. Empirical combination therapy including colistin, a carbapenem, or an aminoglycoside, based on the local resistance epidemiology, might be justified for severely ill patients with suspected infections due to *K. pneumoniae* strains with carbapenemases. Colistin has become the most popular agent for the treatment of infections due to *K. pneumoniae* with

carbapenemases (Pitout, Nordmann and Poirel, 2015). In this study, all the *K. pneumoniae* and *E. coli* KPC-producing strains were sensitive to colistin.

However, in the absence of KPCs, resistance to carbapenems, especially in *K. pneumoniae*, can be granted by the presence of other carbapenamases like VIM, IMP, NDM, OXA-48 and others, or may be linked to different mechanisms like the co-occurrence of permeability defects together with the production of certain β-lactamases, for example AmpC chromosome-encoded cephalosporinases, reduced outer membrane porin OprD expression and associated factors (Iovleva and Doi, 2017).

2.2.2. OXA-48 carbapenemases

Two OXA-48 enzymes were identified in the same patient. In the same pus sample, this enzyme was identified in a *K. pneumoniae* strain and an *E. coli* strain.

OXA-48 carbapenemase, the most relevant in class D β-lactamases, is more prevalent in regions such as North Africa, Turkey and the Middle East, but has also been reported in Europe (Nordmann, 2014). In Portugal, there are still few descriptions of resistance to carbapenems by OXA-48-producing bacteria, and the first OXA-48-producing Enterobacteriaceae was reported in 2014 by Manageiro *et al.* in two different strains of the same patient (*E. coli* and E. *cloacae*).

Similarly to that first description, in this study the OXA-48 enzymes were also found in different strains of the same patient, and since no other cases were identified at CHBV during the timeframe of this study, it highlights the potential of interspecies dissemination of $bla_{\text{OXA-48}}$ gene-harboring plasmids.

According to Pitout, Nordmann and Poirel (2015) different bacteria obtained from the same patient, either as colonizers or as clinical isolates, often contain identical plasmids harboring OXA-48. Plasmids containing $bla_{\text{OXA-48}}$ have the ability to easily and widely disperse between various bacterial species via horizontal transmission. pOXA-48a-like IncL plasmids with Tn*1999.2* and Tn*1999* transposons have been the main sources of the current global distribution of $bla_{\text{OXA-48}}$ into multiple Enterobacteriaceae members (Pitout *et al.*, 2020).

The wide distribution of OXA-48 among different species in hospitals and especially in community settings remains one of the reasons why it is so difficult to limit and control the spread of bacteria with these enzymes (Woerther *et al.*, 2018).

2.2.3. AmpCs and ESBLs

AmpCs and ESBls were detected among the carbapenem-resistant strains analyzed. Between the 5 strains with AmpCs, 2 were isolated from *E. coli* strains, 2 from *E. cloacae* and one from a *S. marcescens* strain.

AmpC β-lactamases are clinically important cephalosporinases, since mediate resistance to commonly used antimicrobial agents, like most penicillins, cephalothin, cefazolin, cefoxitin, and β-lactamase inhibitor-β-lactam combinations. They are very worrisome since can be chromosomally encoded on many Enterobacteriaceae members and also found in plasmids, leading to their easy dissemination. In many bacteria, AmpC enzymes are inducible and can be expressed at high levels by mutation. Overexpression confers resistance to broad-spectrum cephalosporins including cefotaxime, ceftazidime, and ceftriaxone and is a problem especially in infections due to *E. cloacae* and *Enterobacter aerogenes*, where an isolate initially susceptible to these agents may become resistant upon therapy (Mimoz *et al.*, 2012).

Transmissible plasmids have acquired genes for AmpC enzymes, which consequently can now appear in bacteria lacking or poorly expressing a chromosomal bla_{AmpC} gene, such as *E. coli*, *K. pneumoniae*, and *P. mirabilis*. Resistance due to plasmid-mediated AmpC enzymes is less common than ESBL production in most parts of the world but may be both harder to detect and broader in spectrum. AmpC enzymes encoded by both chromosomal and plasmid genes are also evolving to hydrolyze broad-spectrum cephalosporins more efficiently. Sometimes, in non-nosocomial infections they render the antimicrobial empiric therapy improper (Ur Rahman *et al.*, 2018).

Three of the 5 AmpC-producing Enterobacteriaceae were of non-nosocomial origin, despite the difference between nosocomial and non-nosocomial AmpC-producing strains was not accentuated.

Only one ESBL positive strain was detected, in a non-nosocomial *K. pneumoniae* isolate. The most common bacteria carrying ESBL are *Klebsiella* spp. and *E. coli*. 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 (Caínzos, 2008). Nonetheless, in the present study only one ESBL strain was found, this fact does not reflect their distribution within the Hospital. In this study the strain that carries it, is also resistant to the carbapenems, which was the major criteria for the selection of the strains included in this study. This indicates that dispersion of strains fulfilling these two criteria is still low.

It is not reflected in the present study, but previous studies performed in the same hospital showed a high prevalence of ESBL producers (Silva, 2019).

3. Conclusion

Carbapenem resistant isolates have spread globally, which leads to an increasing concern regarding the high morbidity and mortality rates associated with infections caused by these microorganisms. CRE are a major threat, and many of carbapenemases encoding genes are fundamentally associated with plasmids, which contributes to their rapid dissemination among different groups of bacteria and between health units and the community (Ciftci *et al.*, 2019).

In this study was verified that KPC producing *K. pneumoniae* is the most prevalent among CRE. The circulation of OXA-48 enzymes was demonstrated, and it is of particular concern since they have been identified in different bacterial species of the same sample, highlighting their high capacity of dissemination. In addition, carbapenemases producing strains associated with other resistance mechanisms were also observed, namely AmpCs and ESBL.

CRE were considered mostly nosocomial, but their presence in the community is becoming highly accentuated. Therefore, further studies are necessary to understand and determine the origins of these enzymes and their molecular characterization, in order to understand their contributions to epidemiology of carbapenem resistance.

In short, to prevent CRE transmission, is necessary to reinforce infection control measures in the hospital, as well as surveillance of resistant isolates in the hospital settings and the community.

Chapter II. Development of a protocol for manual extraction of nucleic acids

1. Introduction

Antimicrobial susceptibility testing of bacterial pathogens is one of the primary functions of a diagnostic microbiology laboratory once is crucial to for the optimal antimicrobial therapy of patients. This testing is necessary and helpful not only for therapy but also to monitor the spread of resistant organisms or resistance genes throughout the hospital and community.

It is important to have rapid techniques for detecting microorganisms that produce β-lactamases or other similar enzymes. However, the rapid tests are not completely specific.

Molecular diagnosis is of great importance to identify and investigate the presence of resistance genes of interest, and has several advantages: a positive (presence) or negative (absence) answer for a defined resistance determinant; is not dependent upon phenotypic categories such as susceptibility, intermediate susceptibility and resistance for which breakpoints may vary between countries; possibility of detection of very low amounts of the targets of interest that could be difficult to detect using phenotypic methods; and the possibility of be performed directly with clinical specimens, reducing the detection time (Sundsfjord *et al.*, 2004).

Thus, relying on its high sensitivity and specificity, molecular diagnosis is important to generate early assertive results that can be quickly used for the treatment of patients and better infection control, in addition to being also very useful for laboratory research.

However, this genotypic approach also contains certain limitations, once is based on screening for resistance determinants whereas decision making in antimicrobial therapy is preferably based on the detection of susceptibility, and it can only be screen what is already know and genetic methods do not take into account new resistance mechanisms (Woodford and Sundsfjord, 2005).

Genetic methods for the detection of antimicrobial resistance genes and their expression take advantage of the development of nucleic acid amplification techniques. Nucleic acid based detection systems offer rapid and sensitive methods to detect the presence of resistance genes and play a critical role in the elucidation of resistance mechanisms. This is not limited to the detection and identification of microorganisms but is extended to the detection of properties of these microorganisms, such as virulence factors and antimicrobial resistance (Fluit, Visser and Schmitz, 2001).

Taking into account the various advantages and limitations of molecular diagnosis, the genetic approach cannot substitute for phenotypic methods in routine antimicrobial susceptibility testing and it is recommended to use the methodologies in parallel, phenotypic and molecular tests, aiming at a complete and assertive result.

However, in the CHBV microbiology laboratory routine, molecular detection of resistance genes is not implemented, so a protocol that would allow this would be of great interest. Therefore, the objective of this chapter is to implement a protocol for manual extraction of nucleic acids that can be used for further studies, for instance, the identification of resistance genes of interest.

Nonetheless, in the particular case of the section of molecular Biology of CHBV, several tests of molecular biology are already implemented, these tests are automatic and closed, that is to say they aim a specific objective and do not allow variations. Through the implementation protocol of manual extraction of nucleic acids from microorganisms of interest, it would be possible to obtain high quality nucleic acids in minutes, DNA/RNA ready for use in "in house" protocols.

2. Protocol for manual extraction of nucleic acids

This protocol was developed based on the Fosun nucleic acid extraction kit. Considering the situation in the present year of 2020, and the necessary changes in the routine of the Clinical Pathology department of CHBV, instead of bacterial samples, this protocol was performed with SARS-CoV-2 samples.

Before starting the protocol, initial considerations were taken into account, such as do not mix components from different batches; verification of reagents and equipment needed: ethanol, 1.5 mL micro-centrifuge tubes, sterile pipette tips, microcentrifuge for 1.5 mL and 2 mL tubes, metal bath for 1.5 mL and 2 mL tubes.

In the first use, according to table 3, 10 mL of ethanol were pipetted into buffer AW1 concentrate and 40 mL into buffer AW2 concentrate, and 192 mL buffer AVE were pipetted into carrier RNA. The dissolved carrier RNA can be stored at -20ºC for 6 months.

Tabela 3– *Volumes of addition for reagent preparation*.

Components	**Reagent of addition**	**Volumes dof addition**	
		48 tests/kit	**96 tests/kit**
Buffer AW1 concentrate	Ethanol	10 mL	20 mL
Buffer AW2 concentrate	Ethanol	40 mL	40 mL x2
Carrier RNA (lyophilized poder)	Buffer AVE	192 µL	384 µL

Reagent preparation was made by addition of carrier RNA to buffer AVL. As 5 SARS-CoV-2 samples were use, after checking the AVL buffer for precipitate, which was not found, and according to table 4, 2 mL of buffer AVL and 20 µL of carrier RNA were added to and gently mixed by inverting the tube 10 times.

Tabela 4 – *Volumes of buffer AVL and carrier RNA-buffer AVE mix required for the procedure.*

Nº samples	Buffer AVL (mL)	Carrier RNA-AVE (µL)	Nº samples	Buffer AVL (mL)	Carrier RNA-AVE (µL)
1	0.4	4	6	2.4	24
2	0.8	8	7	2.8	28
3	1.2	12	8	3.2	32
4	1.6	16	9	3.6	36
5	2	20	10	4	40

Swab samples were shaken well and rinse. Before starting the protocol, aspects as equilibrate samples to room temperature (15-25ºC) and equilibrate AVE buffer to 70ºC for elution in step 10 were taken in consideration. Sample processing took place according to the following steps:

1. 400 µL prepared AVL buffer containing carrier RNA pipetted into a 1.5 mL microtube.

2. 200 µL of the sample and 20 µL protease K added to the buffer AVL-carrier RNA in the microcentrifuge tube. Vortex for 15 seconds. Sample mixed well with AVL buffer to yield a homogeneous solution, and ensure efficient lysis.

3. Incubated at 70ºC in a methal bath for 10 min.

4. Tube briefly centrifuged to remove drops from inside of the lid.

5. 500 µL of alcohol was add to the sample and mixed by vortex for 15 seconds. After mixing, the tube was briefly centrifuged to remove drops from inside the lid. Sample was mixed thoroughly with the ethanol to yield a homogeneous solution and ensure efficient binding.

6. Carefully added 550 µL of the solution of step 5 to the spin column (in a 2 mL tube) without wetting the rim. Centrifugation was performed at 6000 x g (8000 rpm) for 1 minute, and the filtrate was discarded. The column was opened carefully, and this step was repeated. Each spin column must be closed to avoid cross-contamination during centrifugation.

7. Column was opened carefully and 500 µL of AW1 buffer was added. After closing the cap, centrifugation was performed at 6000 x g (8000 rpm) for 1 minute and the filtrate was discarded.

8. Column was opened carefully and 500 µL of AW2 buffer was added. After closing the cap, centrifugation was performed at 6000 x g (8000 rpm) for 1 minute and the filtrate was discarded. The column was opened carefully, and this step was repeated.

9. Column centrifugation was performed at maximum speed (20,000 x g 14,000 rpm) for 30 seconds to eliminate possible AW2 buffer carryover, and the column was placed in a new 1.5 mL centrifuge tube.

10. With the column placed in a new 1.5 mL microtube, the old collection tube containing the filtrate was discarded. The column was opened carefully and 60 µL of AVE buffer preheated to 70ºC was added in the center of the column. The cap was closed, and the column was incubated at room temperature for 1 minute.

11. Centrifugation at 6000 x g (8000 rpm) for 1 minute was performed and the filtrate is the extracted nucleic acid.

From here, the extracted nucleic acid can be directly detected by PCR or stored at -20ºC.

A version of this protocol for use in the CHBV molecular biology laboratory is available in the annexes (annex 1).

3. Conclusion

As it is known worldwide, in the present year 2020, several changes to the routine had to be made. That was the case in the implementation of this protocol, which was firstly considered to be tested with bacterial strains. However, the pandemic context forced its introduction to be directed to SARS-CoV-2 RNA extraction. Nonetheless it has been tested and optimized in a different context, its implementation was successful.

SARS-CoV-2 RNA was extracted from positive samples and the amplification kit for specific genes used to detect the presence of the virus gave, as expected, positive results. Therefore, nonetheless there was no time to repeat the experiment with bacterial strains, the protocol was implemented and it can be further used in the section of molecular biology of the CHBV.

Chapter III. Development of materials for antibiotic resistance awareness

1. Introduction

Resistance is not a new phenomenon, it was recognized first as a scientific curiosity and later as a threat to effective treatment outcome. Between the 1950s and 1980s, as new families of antimicrobials were developed and modifications of these molecules were achieved, the scientific community believed that we could always remain ahead of the pathogens. However, antibiotic resistance is currently one of the major public health problems of the worldwide recognized by the World Health Organization.

Although antibiotics are necessary and helpful to treat bacterial infections, and in many other situations such before surgeries, agriculture and animal husbandry, the inappropriate use of antimicrobial agents is associated with the emergence of resistance: antimicrobial excessive use is the key driver of resistance, but misuse due to lack of access and underuse due to lack of financial support to complete treatment courses are also factors that lead to this problem (Spellberg *et al.*, 2004).

Improper use occurs both in the hospital environment and in the community in general. In the hospital environment, it is essential to develop approaches to improve the use of antimicrobials, reducing the incidence and dissemination of hospital infections. However, the general public also should be informed of the facts concerning the important role that bacteria play in their lives and well-being, the precious nature of antibiotics and the concomitant importance of using them prudently (Bush *et al.*, 2011). This knowledge can and should be initiated in schools and be available to everyone.

Although microbial resistance is something inevitable, it is within reach of all of us trying to reduce its incidence. Several recommendations have been proposed by different organizations in order to control the spread of resistance and improving antibiotic use are a priority.

The WHO Global Strategy for Containment of Antimicrobial Resistance addresses this challenge. It provides a framework of interventions to slow the emergence and reduce the spread of antimicrobial-resistant microorganisms through: 1) reducing the disease burden and the spread of infection; 2) improving access to appropriate antimicrobials; 3) improving use of antimicrobials; 4) strengthening health systems and their surveillance capabilities; 5) enforcing regulations and legislation; 6) encouraging the development of appropriate new drugs and vaccines. It is a strategy that highlights the aspects of containment of resistance and the need for new research aimed at filling the existing gaps in knowledge and pretends to educate patients and the general community on the appropriate use of antimicrobials and simple measures that may reduce transmission of infection in the household and community (World Health Organization, 2001).

Another initiative is World Antimicrobial Resistance Awareness Week (WAAW). For a week, the priority is to raise awareness of global antimicrobial resistance and encourage good practices in the use of antibiotics among the general public and health workers to reduce the risk of bacteria becoming resistant to them and preserve the effectiveness of antibiotics. This initiative, previously entitled “World Antibiotic Resistance Awareness Week” began in 2015 and in the present year of 2020 the scope of WAAW was expanded, changing its focus from "antibiotics" to the more encompassing term "antimicrobials". Starting in the present year of 2020, WAAW will have a fixed date, from 18 to 24 November (WHO).

In Europe, along with the WAAW, the European Antibiotic Awareness Day (EAAD) is celebrated annually on 18 November. This is a European health initiative coordinated by European Centre for Disease Prevention and Control (ECDC), which provides a platform and support for national campaigns on the prudent use of antibiotics. Due to the COVID-19 pandemic, 2020 EAAD campaign is the first digital campaign and consists of a series of filmed statements by experts, in which, each video addresses a number of key issues related to antimicrobial resistance, addressed to the general public as well as health professionals, whether in hospitals or other health care settings (ECDC).

2. Flyer about antibiotic resistance

In Portugal, one of the examples that fits the initiatives mentioned above is the Association for World Innovation in Science and Health Education (AWISHE). This project has as main objectives promote awareness and training actions about Science and Health subjects, the development educational activities for children, youngsters and adults, create a permanent link with educational and cultural programs, promote the development of national and international Collaborative Learning Communities and promote access to information, educational opportunities, training and development (AWISHE).

With the intention of informing and reminding the general public on this topic, an informational flyer (annex 2) was developed within the scope of the Antibiotic Resistance Awareness Week, which took place from 18 to 24 November 2020, and is available on the AWISHE website.

Being an informative flyer for the general public, it briefly explains what antibiotic resistance is, how the resistance process happens, the main causes and some indications of good antibiotic practices.

3. Conclusion

Antimicrobial resistance remains one of the biggest threats to public health today. As some people may not be aware of this serious problem, it is essential to educate the public in this regard.

Public involvement in the Health and Science content is necessary to contribute to a scientifically formed society, capable of actively participating and supporting current research and innovation challenges. It is important to bring initiatives like campaigns to raise awareness and spread information to the public so that society do not give up on prevent and control antimicrobial resistance.

Apart from these growing initiatives, it is necessary to continue promoting scientific knowledge, by scientists and science communicators, and bring scientific discoveries and updates in the field of antibiotic resistance to everyone.

General Conclusion

Currently, antimicrobial resistance is not only one of the most important global health threats but also one with no easy solution. With the increase in bacterial pathogens resistant to multiple antibiotics, and antimicrobial resistant rates directly related with overuse of antibiotics, efforts to combat antimicrobial resistance focus on concerted attempts to improve diagnosis, antibiotic prescribing practices, and infection prevention strategies.

The results included in this dissertation constitute a study carried out to analyze the epidemiology of β-lactamases in the hospital environment and the community, with emphasis on carbapenemases. Those results reflect that carbapenemase production is the most prominent mechanism underlying carbapenem resistance in Gram-negative pathogens, but other mechanisms such as production of AmpC or ESBL can function together with carbapenemases to confer carbapenem resistance, especially in Enterobacteriaceae.

Infections by MDR microorganisms are a therapeutic challenge, often difficult to overcome. For this reason, in order to improve measures for the prevention and control of nosocomial infection, the molecular diagnosis and genetic study of strains resistant to antibiotics is essential, in the sense of knowing whether we are in the presence of one or several clones, where the gene is located and if it is easily disseminated.

Another major contribution to antimicrobial resistance is the lack of knowledge on the part of the community in general. Scientific communication about antibiotic resistance must be continuously spread among the population, and campaigns to raise awareness on this topic are an asset in the fight against the major problem of antibiotic resistance.

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Annexes

Annex 1: Protocol for Manual Extraction of Nucleic Acids

Protocolo de Extração Manual de Ácidos Nucleicos

Notas iniciais

1. Não misturar componentes de lotes diferentes.
2. O concentrado do tampão AVL e do tampão AW1 contém sal caotrópico, que é irritante.
3. Reagentes e equipamento fornecidos pelo utilizador:
    a. Álcool
    b. Microtubos de 1,5 mL
    c. Pontas de pipetas estéreis
    d. Microcentrífuga para tubos de 1,5 mL e 2 mL
    e. Banho seco para tubos de 1,5 mL e 2 mL
4. Na primeira utilização, pipetar o álcool para o concentrado do tampão AW1 e AW2, e pipetar o tampão AVE para o carrier RNA de acordo com a tabela 1. O carrier RNA dissolvido deve ser armazenado a -20ºC durante 6 meses.

Tabela 1 – *Volumes de adição para preparação de reagentes.*

Componentes	**Reagente de adição**	**Volumes de adição**	
		48 testes/kit	**96 testes/kit**
Concentrado do tampão AW1	Álcool	10 mL	20 mL
Concentrado do tampão AW2	Álcool	40 mL	40 mL x2
Carrier RNA (pó liofilizado)	Tampão AVE	192 µL	384 µL

Protocolo

1. Preparação dos reagentes

1.1. Adição do Carrier RNA ao Tampão AVL

Verificar o tampão AVL quanto a precipitado e, se necessário, incubar a 70ºC até que o precipitado esteja dissolvido. Calcular o volume da mix tampão AVL-carrier de RNA necessária por quantidade de amostras, selecionando o número de amostras a serem processadas simultaneamente da tabela 2. Para números maiores de amostras, os volumes podem ser calculados usando a seguinte fórmula:

n x 0,4 mL = y mL
y mL x 4 µL/mL = z µL

Em que: n = número de amostras a serem processadas simultaneamente
y = volume calculado de tampão AVL
z = volume de carrier RNA-tampão AVE a adicionar ao tampão AVL

Misturar ao inverter o tubo 10 vezes. Para evitar fazer espuma, não usar o vórtex.

Tabela 2 – *Volumes do tampão AVL e mix carrier RNA-tampão AVE necessários para o procedimento.*

Nº de amostras	Tampão AVL (mL)	Carrier RNA-AVE (µL)	Nº de amostras	Tampão AVL (mL)	Carrier RNA-AVE (µL)
1	0,4	4	6	2,4	24
2	0,8	8	7	2,8	28
3	1,2	12	8	3,2	32
4	1,6	16	9	3,6	36
5	2	20	10	4	40

1.2. Aspetos a verificar antes de começar o protocolo

Manter as amostras à temperatura ambiente (15-25ºC).

Manter o tampão AVE a 70ºC para a eluição no passo 3.10.

Verificar se o tampão AW1 e o tampão AW2 foram preparados de acordo com as instruções do passo 1.1.

Adicionar o carrier RNA reconstituído no tampão AVE ao tampão AVL de acordo com as instruções da tabela 1.

2. Processamento das amostras

Amostras de sangue ou plasma não precisam de ser processadas.

Amostras de zaragatoas: agitar bem e lavar (se for necessário conservar durante muito tempo, transferir a solução de para um tubo de 1,5 mL e armazenar a solução entre -20ºC a -70ºC).

3. Procedimento

3.1. Pipetar 400 µL do tampão AVL preparado com carrier-RNA num microtubo de 1,5 mL.

3.2. Adicionar 200 µL da amostra e 20 µL da protease K ao tampão AVL-carrier RNA no microtubo. Misturar no vortex durante 15 segundos.

Nota: Para assegurar uma lise eficiente, é essencial que a amostra seja bem misturada com o tampão AVL para produzir uma solução homogénea. Amostras congeladas que só foram descongeladas uma vez também podem ser usadas.

3.3. Incubar a 70ºC em banho seco durante 10 min.

Nota: A lise das partículas virais está completa após lise durante 10 min a 70ºC. Tempos de incubação mais longos não afetam o rendimento ou a qualidade do RNA purificado.

3.4. Centrifugar brevemente o tubo para remover gotas da tampa.

3.5. Adicionar 500 µL de álcool (etanol anidro) à amostra e misturar no vortex durante 15 segundos. Após misturar, centrifugar brevemente para remover gostas da tampa do microtubo.

Nota: Usar apenas etanol anidro, uma vez que outros álcoois podem resultar em redução do rendimento e pureza do RNA. Não usar álcool desnaturado, que contém outras substâncias como metanol ou metiletilcetona. Para garantir uma ligação eficiente, é essencial que a amostra seja bem misturada com o etanol para produzir uma solução homogénea.

3.6. Cuidadosamente adicionar 550 µL da solução do passo 3.5 à coluna (num tubo de 2 mL) sem molhar a borda. Fechar a tampa e centrifugar a 6000 x g (8000 rpm) durante 1 minuto, e descartar o filtrado. Abrir a coluna cuidadosamente e repetir este passo mais uma vez.

Nota: Fechar cada coluna para evitar contaminação cruzada durante a centrifugação.

Nota: A centrifugação é realizada a 6000 x g (8000 rpm) para limitar o ruído da microcentrífuga. A centrifugação à maior velocidade não afetará o rendimento ou a pureza do RNA viral. Se a solução não tiver passado completamente pela membrana, centrifugar novamente a uma velocidade mais elevada até que toda a solução tenha passado pela membrana.

3.7. Abrir a coluna cuidadosamente e adicionar 500 µL do tampão AW1. Fechar a tampa e centrifugar a 6000 x g (8000 rpm) durante 1 minuto, e descartar o filtrado.

3.8. Abrir a coluna cuidadosamente e adicionar 500 µL do tampão AW2. Fechar a tampa e centrifugar a 6000 x g (8000 rpm) durante 1 minuto, e descartar o filtrado. Abrir a coluna cuidadosamente e repetir este passo novamente.

3.9. Centrifugar a coluna à velocidade máxima (20 000 x g 14 000 rpm) durante 30 segundos para eliminar possível passagem do tampão AW2, e colocar a coluna num novo tubo de 1,5 mL.

3.10. Colocar a coluna num novo microtubo de 1,5 mL. Descartar o antigo tubo de colheita que contém o filtrado. Abrir a coluna cuidadosamente e adicionar 60 µL do tampão AVE pré-aquecido a 70ºC no centro da coluna. Fechar a tampa e incubar a temperatura ambiente durante 1 minuto.

3.11. Centrifugar a 6000 x g (8000 rpm) durante 1 minuto. O filtrado é o ácido nucleico extraído, que pode ser diretamente detetado por PCR ou guardado a -20ºC.

Nota: Uma única eluição com 60 µL de tampão AVE é suficiente para eluir pelo menos 90% do ácido nucleico da coluna. Realizar uma dupla eluição usando 2 x 40 µL de tampão AVE vai aumentar o rendimento até 10%. Eluição com volumes inferiores a 30 µL levará a baixos rendimentos e não aumentará a concentração final de ácido nucleico no eluído. O ácido nucleico eluído é estável durante um ano quando armazenado a -20ºC ou -70ºC.

Annex 2: Antibiotic Resistance Awareness Week Flyer

Front page

Semana da sensibilização para a **RESISTÊNCIA A ANTIBIÓTICOS**

18 a 24 de novembro de 2020

CAUSAS DA RESISTÊNCIA AOS ANTIBIÓTICOS

- Prescrição a mais de antibióticos por parte da comunidade médica
- Uso desnecessário de antibióticos na agricultura
- Controlo de infeção insuficiente nos hospitais e clínicas
- Uso incorreto dos antibióticos por parte dos utentes

Tomar antibióticos desnecessariamente aumenta o risco das bactérias adquirirem resistência!

Portugal é um dos países que apresenta maior taxa de resistência aos antibióticos, o que está relacionado com a sua utilização indevida.

O que deve fazer:

- Não se auto medique, apenas tome antibióticos quando receitados pelo seu médico;
- Não pressione o seu médico nem farmacêutico para lhe receitar antibióticos;
- Não utilize sobras de antibióticos utilizados para outras situações ou de outras pessoas;
- Tome sempre os antibióticos até ao fim.

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