Universidade de Aveiro Departamento de Biologia
Ano 2019

LILIANA INÁCIO AZEVEDO

EPIDEMIOLOGY OF STRAINS RESISTANT TO COLISTIN

EPIDEMIOLOGIA DE ESTIRPES RESISTENTES À COLISTINA

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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, e coorientação do Doutor Elmano José da Cruz Ramalheira, Diretor do Serviço de Patologia Clínica do Centro Hospitalar do Baixo Vouga, E. P. E, Aveiro.

A ti mano, o melhor do mundo.

o júri

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agradecimentos

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palavras-chave

Resistência antimicrobiana, gene *mcr-1*, infeções complicadas do trato urinário, cultura cientifica.

resumo

As infeções bacterianas continuam a ser uma das mais preocupantes causas de mortalidade e morbilidade, a nível global. O uso excessivo e inadequado de antibióticos em áreas tão diversas como a veterinária, a agricultura e a nível hospitalar contribuiu para o aumento da disseminação de estirpes bacterianas multirresistentes (MDR). Infeções complicadas do trato urinário (cUTIs) podem ser nosocomiais ou adquiridas na comunidade. O aumento dos determinantes de resistência antimicrobiana, bem como a falta de desenvolvimento de novos compostos leva a uma restrição de opções terapêuticas para estas doenças infeciosas.

O objetivo deste estudo passa por avaliar a epidemiologia da resistência à colistina, bem como a presença do gene *mcr-1* em estirpes de pacientes do Centro Hospitalar do Baixo Vouga E.P.E (CHBV), durante setembro de 2017 a maio 2019. Devido à falta de novos antimicrobianos, avaliou-se também a atividade do composto Ceftolozano/tazobactam (Cef/taz) para cUTIs severas, causadas por membros da família Enterobacteriaceae produtores de ESBL e *Pseudomonas aeruginosa* MDR. Adicionalmente, este trabalho conta com a participação num projeto de Comunicação de Ciência e Saúde através da Educação.

Os resultados obtidos demonstraram que o gene *mcr-1* foi detetado em dois (estirpe *E. coli* e *K. pneumoniae*) dos 13 isolados recolhidos resistentes à colistina. O Cef/taz demonstrou elevada atividade para os isolados testados, pois de 67, apenas quatro apresentaram um fenótipo intermédio e três um fenótipo resistente, sendo que dois destes foram *P. aeruginosa* XDR. Relativamente à comunicação de Ciência e Saúde, nomeadamente sobre resistência antimicrobiana, através da Educação, para os 25 alunos do Agrupamento de Escolas de Oliveirinha, esta teve uma grande recetividade por parte dos mesmos. Cerca de 70% dos resultados do questionário desenvolvido, foram positivos.

Em suma, este trabalho enfatiza a emergência de estirpes resistentes em diferentes ambientes, mas principalmente a nível hospitalar, até para os antibióticos usados como último recurso. Assim, é notório a necessidade de uma contínua vigilância e monitorização epidemiológica da resistência antimicrobiana, bem como o contribuir para uma comunidade consciente e proativa.

keywords

Antimicrobial resistance, *mcr-1* gene, complicated urinary tract infections, scientific culture

abstract

Bacterial infections continue to be one of the most worrying causes of mortality and morbidity worldwide. The excessive and inappropriate use of antibiotics in diverse areas as veterinary, agriculture and Hospital has contributed to the increase of the spread of multi-resistant (MDR) bacterial strains. Complicated urinary tract infections (UTIs) can be nosocomial or community acquired. The increase of the antimicrobial resistance determinants, as well as the paucity of development of new compounds leads to a narrowing of therapeutic options for these infectious diseases.

The aim of this study was to evaluate the epidemiology of resistance to colistin and the presence of the *mcr-1* gene in strains from patients attending the "Centro Hospitalar do Baixo Vouga, E.P.E", (CHBV) during September 2017 to May 2019. Due to the lack of new antimicrobial agents, the activity of the Ceftolozan/tazobactam (Cef/taz) for severe cUTIs caused by members of Enterobacteriaceae family ESBLs producers and *Pseudomonas aeruginosa* MDR was also evaluated. Additionally, this work counts with a participation in a project of Science and Health Communication through Education.

The results obtained demonstrated that the *mcr-1* gene was detected in two (*E. coli* and *K. pneumoniae* strains) of the 13 colistin-resistant isolates collected. The Cef /taz showed high activity for the tested isolates, since of 67, only four presented an intermediate phenotype and three a resistant phenotype, two of which were *P. aeruginosa* XDR. Regarding the Communication of Science and Health, namely about antimicrobial resistance, through Education, for the 25 students of the "Agrupamento de Escolas de Oliveirinha", this one had a great receptivity on the part of the same ones. About 70% of the results of the questionnaire developed were positive.

In summary, this work emphasizes the emergence of resistant strains in different environments, but mainly at the hospital setting, even for the antibiotics used as a last resource. Thus, the need for continued surveillance and epidemiological monitoring of antimicrobial resistance is well known, as well as contributing to a conscious and proactive community.

Index

List of Publications

This thesis includes results presented in the following publications:

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

Liliana Azevedo, Ana Rita Silva, Patrícia Matos, Marta Tacão, Isabel Henriques, Elmano Ramalheira, Sónia Ferreira. ECCMID 2019: 29th European Congress of Clinical Microbiology & Infectious Diseases in Amsterdam, Netherlands, from 13-16 April 2019 – (Chapter 1)

(Appendix 1)

PEMI: Mobilidade de ideias no caminho da inclusão

Inês Cravo Roxo, Ana Santos-Carvalho, Ana Rita Silva, Daniela Meireles, João Borges, Joel Pinto, Liliana Azevedo, Patrícia Matos, Patrícia Quitério, Paulo Almeida, Rafaela Araújo, Richard Marques, Rui Soares, Susana Alarico, Sónia Ferreira. SciComPT 2019: Rede de Comunicação de Ciência e Tecnologia de Portugal, Aveiro, Portugal, 30-31 maio 2019 – (Chapter 3)

(Appendix 2)

O (ir)resistível mundo dos antibióticos

Liliana Azevedo, Ana Rita Silva, Patrícia Matos, Inês Cravo Roxo, Sónia Ferreira. SciComPT 2019: Rede de Comunicação de Ciência e Tecnologia de Portugal, Aveiro, Portugal, 30-31 maio 2019 – (Chapter 3)

(Appendix 3)

Abbreviations

AMC - Amoxicillin/clavulanic acid;
AMK - Amikacin;
AMP - Ampicillin;
AST - Antimicrobial susceptibility test;
AWISHE - Association for World Innovation in Science and Health Education;
CAZ - Ceftazidime;
Cef/taz - Ceftolozane/tazobactam;
CHBV - "Centro Hospitalar do Baixo Vouga, E.P.E";
CHUC - "Centro Hospitalar e Universitário de Coimbra";
CIP - Ciprofloxacin;
CLED - Cystein Lactose Electrolyte Deficient medium;
COZ - Cefuroxime;
CPE - Carbapenemases-producing Enterobacteriaceae;
CTX - Cefotaxime;
cUTI - complicated Urinary-tract infections;
ER - Emergency room;
ERIC - Enterobacterial Repetitive Intergenic Consensus;
ERT - Ertapenem;
ESBLs - Extended-spectrum β-lactamases;
FEB - Cefepime;
FOS - Fosfomycin;
GEN - Gentamycin;
HAI - Healthcare-associated infections;
HGT - Horizontal gene transfer;
IAI - Intraabdominal infections;
ICATE - Infection Control Awareness Through Education;
ICU - Intensive care units;
KPC - *K. pneumoniae* carbapenemase;
LPS - Lipopolysaccharide;
MBLs - Metallo-β-lactamases;
MDR - Multidrug resistant;
MED - Medicine;
MEM - Meropenem;
MGEs - Mobile genetic elements;
MIC - Minimal inhibitory concentration;
NIT - Nitrofurantoin;
ORT - Orthopedics;
PBP - Penicillin-binding proteins;
PCR - Polymerase Chain Reaction;

PDR - Pan drug resistant;
PEMI - “Projeto de Estímulo à Mobilidade de Ideias”;
REP - Repetitive element palindromic;
SUR - Surgery;
SxT - Cotrimoxazole;
TZP - Piperacillin/tazobactam,
UTI - Urinary tract infection;
WHO - World Health Organization;
XDR - Extensively drug resistant;

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1 General Introduction

1.1 Healthcare-associated infection

There is a common etiology of healthcare-associated infections (HAI), such as urinary tract, intraabdominal infections and nosocomial bloodstream infections. According to European point prevalence survey conducted by the European Center for Disease Prevention and Control, the urinary tract infections (UTIs) accounted for 19.0% of all HAI (Khoshnood et al. 2017). The type of etiological pathogens causing HAI differs between regions and hospitals and actually it may compromise advanced medicine (Potron et al. 2015; Pfaller et al. 2017; Saran et al. 2019).

Multidrug resistant (MDR) Gram-negative bacteria are spreading fast and stealthily globally, these bacteria have become prevalent in hospitals being at the origin of severe infections. This fact is of special concern because it may associate resistance to the three main classes of antibiotics in a single isolate as the result of the combination of different mechanisms or the action of a single potent resistance mechanism (Sader et al. 2014a; Nordmann and Poirel 2016). Now, one is facing infections caused by extensively drug resistant (XDR) bacteria that are resistant to most antimicrobial agents and also pan drug resistant (PDR) bacteria that are resistant to all antimicrobial agents available for clinical use (Magiorakos et al. 2012; Sader et al. 2014b; Kaye and Pogue 2015; Escolà-Vergé et al. 2018; Saran et al. 2019). Figure 1 shows the relationship between MDR, XDR and PDR bacteria.

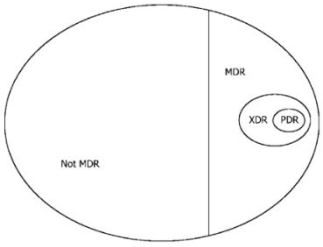


Figure 1 Schematic representation of the relationship between MDR, XDR and PDR bacteria (adapted from (Magiorakos et al. 2012)).

A significant portion of the resistant bacterial species seen in hospitalized patients are the ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacter species. All of these pathogens have intrinsic resistance to one or more classes of antibiotics (Wyres and Holt 2018). Between MDR or even PDR bacteria, *A. baumannii* and *P. aeruginosa* are the most commonly identified and they cause about one-third of HAI. In addition, *E. coli* and

K. pneumoniae accounting for 27% of all pathogens and 70% of all Gram-negative pathogens causing HAI (Kaye and Pogue 2015; Theuretzbacher 2017).

HAI due to these microorganisms and inappropriate initial antibiotic therapy leads to increasing hospital length of stay and consequently to an increase of costs as well as mortality and morbidity rates. In the hospital environment, antimicrobial resistance is attributed to the inappropriate and indiscriminate use of antibiotics in patients highly susceptible such as immunocompromised patients. This, together with deficient infection control measures highlight the need for new effective therapies and new control measures for these microorganisms (Potron et al. 2015; Tato et al. 2015; Díaz-cañestro et al. 2018; Morehead and Scarbrough 2018; Wyres and Holt 2018).

1.2 Urinary tract infections

According to WHO in Europe, UTIs are one of the most common bacterial infections hospital wide (27%), affecting 150-250 million people of all ages (Flores-Mireles et al. 2015; Khoshnood et al. 2017). The urinary tract is a common source of life-threatening infections, and an important cause of sepsis in patients admitted to hospital wards, emergency rooms (ER), and intensive care units (ICU) (Levy et al. 2012). UTI is an infection anywhere in the urinary tract (urethra, bladder, ureters, or kidneys) that triggers an inflammatory reaction by the host (Foxman 2014). The development of UTI is dependent on both host and microbiological factors (Foxman 2014; Walsh and Collyns 2017). Women are especially prone to UTIs because of their shorter urethral length and frequent vaginal and periurethral colonization. Other risk factors include anatomic and functional urologic abnormalities, sexual activity, history of UTIs (especially age <15 years), urinary incontinence, and physical limitations. An estimated 11% of women report at least one physician-diagnosed UTI per year, and 20-30% report multiple recurrences. The prevalence of UTI increases with age. In women over 65 years of age, UTIs increase by approximately 20% (Chu and Lowder 2018).

1.2.1 Classification of UTI

Clinically, there are different types of UTIs with different severity like cystitis, urethritis, acute urethral syndrome, pyelonephritis, significant bacteriuria, asymptomatic bacteriuria, pyuria, prostatitis, and urosepsis. These infections are categorized as uncomplicated or complicated (Cek et al. 2014; Walsh and Collyns 2017). Uncomplicated

UTIs typically affect individuals who are otherwise healthy without urinary tract abnormalities. Complicated UTI (cUTI), the most severe type of UTI, is a symptomatic infection of the bladder or kidney, which normally occur in the presence of a structural or functional abnormality of the genitourinary tract (Wagenlehner et al. 2015; Walsh and Collyns 2017; Chu and Lowder 2018). UTIs are differentiated into lower UTIs, which occurs in the bladder, such as cystitis, and upper UTIs that occur in the kidneys, like pyelonephritis, as shown in Figure 2 (McLellan and Hunstad 2016; Chu and Lowder 2018). In this case, urinary symptoms may or may not be present (Foxman 2014).

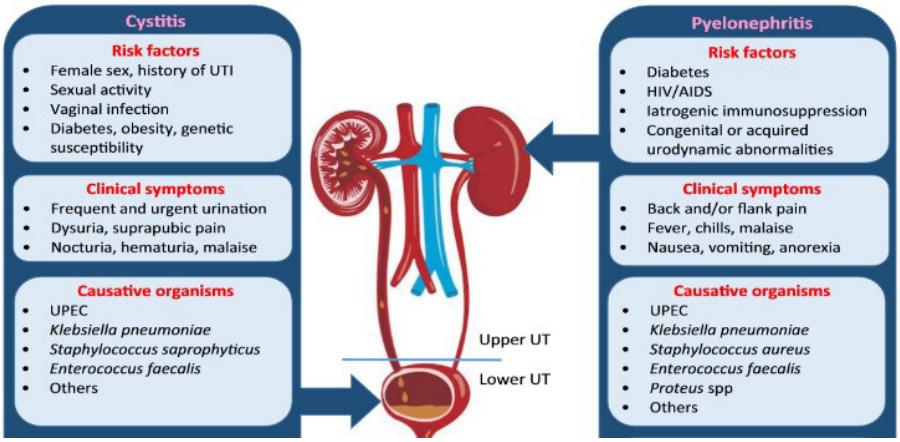


Figure 2 Risk factors, clinical symptoms and causative organism of one lower UTI – cystitis and one upper UTI – pyelonephritis (adapted from (McLellan and Hunstad 2016)).

1.2.2 Signs and symptoms

Signs and symptoms of UTIs depend on the intensity of the inflammatory response and differ according to the local of infection (Walsh and Collyns 2017). The most common symptoms are frequent and urgent urination and dysuria. Systemic symptoms, such as nausea, vomiting, flank pain, upper back pain, and fevers may indicate ascension of infection to the upper urinary tract and, in these cases shouldn't be treated as uncomplicated UTI (Chu and Lowder 2018).

1.2.3 Main strains – etiopathogenesis

In most cases, the colonizing bacteria do not cause disease, because the host uses the immune response and urination to quickly remove the bacteria from the system. When the host is compromised, a very wide range of organisms including viruses, bacteria, fungi, and

parasites can potentially infect the urinary tract. Bacteria that do cause UTI are able to survive in the urinary tract because they developed special features (e.g., biofilm formation, urothelial cell invasion, adhesins, toxins, and siderophores) or inhabit a compromised host, that doesn't have the ability to remove them (e.g., catheterization) (Foxman 2014). These microorganisms may reach the bladder via hematogenous or lymphatic but the most common bacterial causes of UTI are the uropathogens from fecal flora that colonize the gut, because they can reach the urinary tract through colonization of the peri-urethral region (Khoshnood et al. 2017; Walsh and Collyns 2017; Chu and Lowder 2018). Uncomplicated UTIs are mostly caused by Gram-negative bacteria, namely Enterobacteriaceae family, 70%–80% by uropathogenic *E. coli*. Species that cause cUTI, with varying frequency, are *Klebsiella* spp., *P. aeruginosa*, *Proteus* spp., *Morganella morganii* and *Providencia stuartii* and the Gram-positive *Enterococcus* spp., *Streptococcus agalactiae*, *Staphylococcus saprophyticus,* and methicillin-resistant *Staphylococcus aureus* (Khoshnood et al. 2017; O'Grady et al. 2018). Species such as *Enterobacter*, *Serratia,* and *Citrobacter* rarely cause UTIs in normal hosts but commonly cause healthcare-associated UTIs, notably in the presence of an indwelling catheter (Walsh and Collyns 2017).

1.2.4 Risk factors

Prolonged urinary catheter usage is a risk factor for UTI, largely due to the ability of bacteria to establish a biofilm on the catheter that resists clearance by host defense and antimicrobial agents. Catheter-associated UTIs represent the most common nosocomial infections and it increased hospital length of stay, morbidity, and mortality (McLellan and Hunstad 2016). Besides the use of a catheter, there are other risk factors such as sexual activity, vaginal infection, genetic susceptibility/anatomic abnormalities, diabetes and obesity (Foxman 2014; Walsh and Collyns 2017). Figure 3 shows the percentage of microorganisms that cause the two types of UTIs and the main risk factors associated.

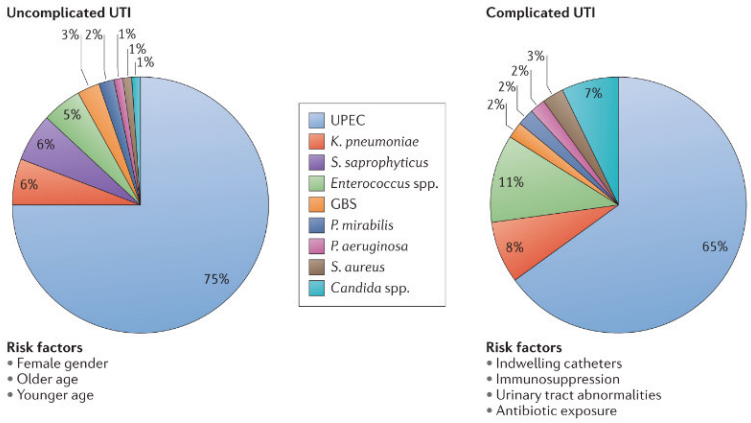


Figure 3 Epidemiology and the most common risk factors of two types of UTIs (adapted from (Flores-Mireles et al. 2015)).

1.2.5 Treatment

Current therapeutics are suboptimal because antibiotics only eliminate bacteriuria transitorily and their administration neither decreases the frequency of symptomatic infection nor prevents further episodes of asymptomatic bacteriuria. These recalcitrant infections can become a significant health problem and diminish the quality of life for patient (Foxman 2014; McLellan and Hunstad 2016). Drugs commonly recommended for uncomplicated UTIs include cotrimoxazole, nitrofurantoin, cephalexin, and ceftriaxone. The fluoroquinolones, such as ciprofloxacin and levofloxacin, followed by cephalosporins, aminoglycosides, and penicillins are commonly recommended for cUTIs (Cek et al. 2014; Wagenlehner et al. 2015).

Using a limited number/class of antibiotics, especially fluoroquinolones, cephalosporins, and penicillins, clearly creates pressure for the selection of resistant pathogens such as MDR microorganism as collateral damage (Cek et al. 2014; Vazquez and Ampuero 2017; Chu and Lowder 2018). For the treatment of UTI caused by these microorganisms, it is suggested either monotherapy or bitherapy, and should be decided considering the severity of underlying conditions, the severity of infection, minimal inhibitory concentration (MIC) values and clinical response (Vazquez and Ampuero 2017).

Given the current high levels of antimicrobial use for the treatment of suspected UTIs, it's likely that the aetiological agents represent an important reservoir of antimicrobial resistance genes, which vary with infected population and geographic region (Foxman 2014;

O'Grady et al. 2018). It's important to highlight that it's not recommended to administer empirical antimicrobial treatment with antibiotics having more than 20% of resistant strains for uncomplicated UTI or 10% for complicated ones (Vazquez and Ampuero 2017; O'Grady et al. 2018).

1.3 Microorganisms

1.3.1 Enterobacteriaceae family

Enterobacteriaceae family is formed by Gram-negative bacilli that share some characteristics: nonspore-forming, are glucose fermenters (often with gas production), facultative anaerobes, oxidase-negative, catalase-positive, and reduce nitrates to nitrites. *E. coli, Klebsiella* spp., *Proteus* spp., *Morganella* spp., *Providentia* spp., *Enterobacter* spp., *Serratia* spp, *Salmonella* spp., *Shigella* spp. and *Yersinia* spp. are some of the Enterobacteriaceae members (Church 2015).

These species live in the environment and some of them, in the gut, where they don't harm, but, sometimes, they can appear in body sites where normally, doesn't exist bacteria (e.g. bladder or blood) and cause infections. This can occur especially in immunocompromised patients who are made vulnerable by underlying disease, injury or long periods of hospitalization, mostly in the ICU (Theuretzbacher 2017; Hawkey et al. 2018; Morehead and Scarbrough 2018; Wyres and Holt 2018).

According to the recent reports of United States National Health Care Safety Network, this family is responsible for 30% of HAIs, like intraabdominal infections (IAIs), UTIs (45%), ventilator-associated pneumonia, and bacteremia (Kaye and Pogue 2015; Khoshnood et al. 2017; Saran et al. 2019). *E. coli* is the most prevalent microorganism causing UTIs and *K. pneumoniae* is the most common pathogen in bloodstream infections (Khoshnood et al. 2017; Morehead and Scarbrough 2018; Lykholat 2018).

The majority of Enterobacteriaceae family produce plasmid-encoded enzymes - β-lactamases, namely extended-spectrum β-lactamases (ESBLs), AmpC enzyme (cephalosporinases) and carbapenem-hydrolyzing β-lactamases (carbapenemases) (Sultan et al. 2018). *K. pneumoniae* strains have different genes of antimicrobial resistance that have been identified over the years as it is chronologically represented in Figure 4. The *mcr*-1 gene

(mobile colistin resistance), which confers colistin resistance is the most recent, but it already represents a worrying public health problem (Cannatelli et al. 2014; Poirel et al. 2017).

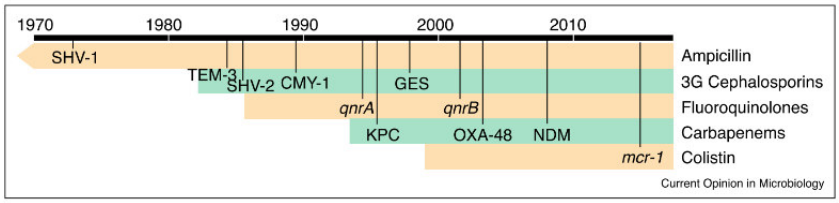


Figure 4 Timeline of mobile antimicrobial resistance detected in *K. pneumoniae* and respective antibiotic class resistance (adapted from (Wyres and Holt 2018)).

1.3.1 *Pseudomonas aeruginosa*

P. aeruginosa is aerobic Gram-negative bacteria, oxidase-positive, from the Pseudomonadaceae family, ubiquitous in the environment, including the hospital environment. It's an opportunistic and lethal bacteria responsible for greater than 50,000 infections per year and ~8% of all HAI, namely ventilator-associated pneumonia and it's the second most common organism causing catheter-associated UTIs (Kaye and Pogue 2015; Morehead and Scarbrough 2018).

This microorganism has innately high environmental tolerance, since it has an almost impenetrable outer membrane, and the capacity to form biofilms gives them the capacity to resist to the majority of antimicrobial agents (Morehead and Scarbrough 2018). Approximately 25-50% of *P. aeruginosa* isolates are resistant to many antibiotics available and the accumulation of various chromosomal mutations lead to the emergence of MDR strains (up to 10-50%) (Cabot et al. 2014; Walsh and Collyns 2017)

The immediate initiation of a correct therapy is essential: a delay in the treatment is associated with increased morbidity and mortality in patients with severe *P. aeruginosa* infections, since these pathogens are extremely well adapted to the hospital environment (Sader et al. 2014b; Pfaller et al. 2017; Giacobbe et al. 2018).

1.4 Antibiotics

An antimicrobial compound is a substance, naturally or artificially obtained, with the capability of killing – bactericidal - or inhibiting microorganism growth - bacteriostatic, active against bacteria, fungi, and parasites associated with a MIC value (El Salabi et al. 2013). Antibiotics emerged as a powerful tool in counteracting infectious diseases, following

the accidental discovery of penicillin from *Penicillium notatum* by Alexander Fleming in 1924 (Fleming 1929; Sultan et al. 2018). However, it was only in 1940 that penicillin was used to treat human diseases (Chellat et al. 2016).

The antimicrobial agents can penetrate the outer membrane through the cytoplasmic membrane or by lipid-mediated pathway and general porin diffusion. In Gram-negative bacteria, hydrophobic agents used the lipid-mediated pathway whereas the hydrophilic antibiotics use porins to reach their target. The ideal antibiotic should produce this effect with a small amount, without toxic or collateral effects for the human host (El Salabi et al. 2013; Sultan et al. 2018). Antibiotics, used commonly in the treatment and prevention of infections, are classified in β-lactams, Tetracyclines, Aminoglycosides, Macrolides, Sulfonamides, Quinolones, Diaminopyrimidines, and Polymyxin according to their structure and degree of affinity to target (Sultan et al. 2018).

1.4.1 Mechanisms of action and antibiotic resistance

Different classes of antibiotics can have different mechanisms of action, such as affecting cell wall synthesis (β-lactams), inhibition of protein synthesis (Tetracycline, Chloramphenicol, Aminoglycosides, Macrolides), interference with nucleic acid synthesis (Rifampicin, Quinolones), interfering with metabolic pathways (Folic acid analogs, Sulfonamides) and disrupting cell membrane structure (Polymyxins). Antibiotics are specific in their effect toward different bacterial species.

According to the World Health Organization (WHO) resistance to antibiotics is the reduction in effectiveness of a drug, and it occurs when bacteria multiply and adapt in their presence (Shaikh et al. 2015; Sultan et al. 2018).

There is a huge abundance of resistance phenotypes in bacteria but the mechanisms of resistance to any antibiotic are only five, divided in biochemical and genetic aspects: enzymatic degradation of the antibiotic by hydrolysis or modification, modification of the antibiotic target, reduction of membrane permeability to antibiotics either by decreasing uptake or increasing efflux, prevention of absorption or intake of the antibiotic and mutations (Shaikh et al. 2015; Mo et al. 2018; Sultan et al. 2018). These mechanisms can be chromosomal or mediated by mobile genetic elements (MGEs) such as plasmids, transposons or integrons. Thus, when the genes encoding antimicrobial resistance determinants are in the chromosome, they are inherited by the daughter cells, and therefore named intrinsic

resistance, which is a natural phenomenon, and it's exhibited by all members of the species. When these genes might be horizontally transmitted on MGEs, the mechanism of resistantce developed is called acquired resistance. The MGEs transmitting resistance genes can be acquired only by a few members of a given species, so, the resistant phenotype isn't present in all members of same specie (Codjoe and Donkor 2017; Koulenti et al. 2019).

Gram-negative bacteria have the capacity to resist to different antibiotics since they can exhibit several mechanisms of resistance. A common mechanism is the DNA alteration (Kaye and Pogue 2015; Tato et al. 2015). It can occur via successive mutations or recombination, under selective pressure that is imposed by antimicrobial compounds or other contaminants, such as biocides or heavy metals. In other cases, this may occur by horizontal gene transfer (HGT), usually via MGEs, that act as vehicles for resistance genes acquisition and their successive propagation (Hawkey et al. 2018; Wyres and Holt 2018; Koulenti et al. 2019). HGT is the principal mechanism for the spread of antibiotic resistance because of their capacity to relocate DNA between host genomes via conjugation, transduction, or transformation (Shaikh et al. 2015; Chellat et al. 2016; Sultan et al. 2018). Figure 5 summarizes the action of antibiotics on the different targets as well as the different mechanisms of resistance developed by bacteria.

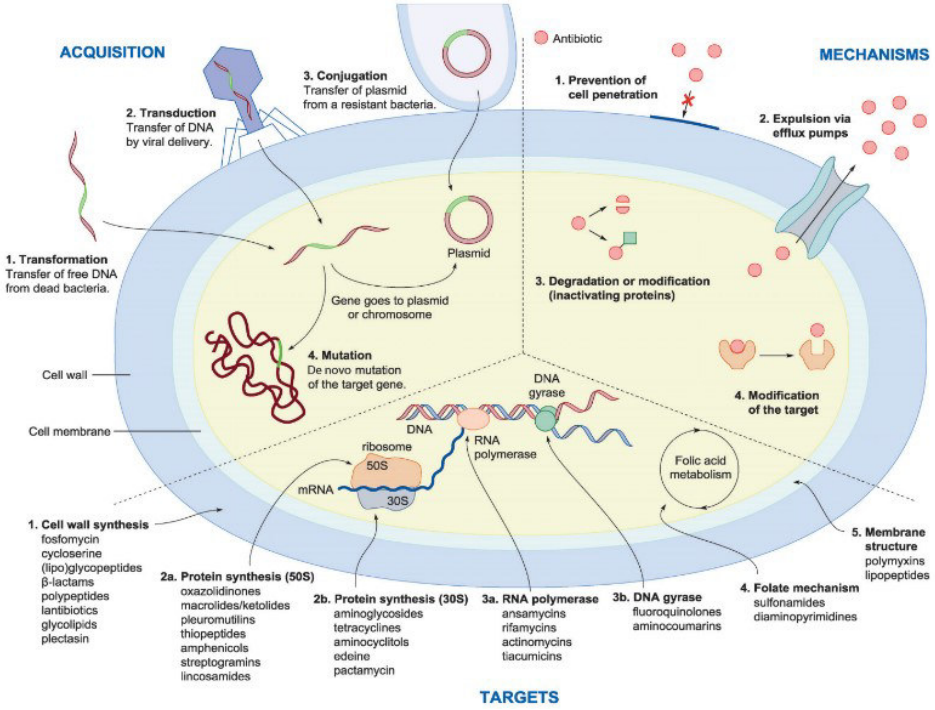


Figure 5 The acquisition pathways, the main bacterial mechanisms of resistance, and the antibiotics targets (adapted from (Chellat et al. 2016)).

β-lactam antimicrobial agents were introduced into the clinic in the 1940s exemplified by the antibiotic penicillin (Fleming 1929). To this day, β-lactams are still the most common widely used around the globe to treat infections caused by human pathogenic bacteria (El Salabi et al. 2013; Chellat et al. 2016)

1.4.2 β-lactams

β-lactam are bactericidal agents that interrupt bacterial cell wall synthesis through of covalent binding to Penicillin-binding proteins (PBPs) once they act as a competitive antagonist. PBPs bind to β-lactam and aren't no longer available for their natural substrate, so, the terminal steps of peptidoglycan cross-linking in both Gram-negative and Gram-positive bacteria doesn't occur (Bush and Bradford 2016; Chellat et al. 2016).

This class comprehends penicillins, cephalosporins, carbapenems, monocyclic β-lactams and β-lactamases inhibitors, which are represented in Figure 6. Different subclasses of β-lactam have different affinities for the different PBPs (Llarrull et al. 2010).

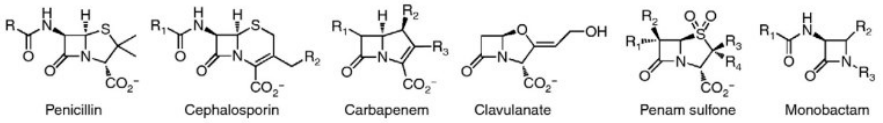


Figure 6 The structures of the β-lactam family (adapted from (Llarrull et al. 2010)).

1.4.3 Mechanisms of resistance to β-lactams

Enzymatic degradation, PBP acquisition, mutations, efflux pumps, and porin losses are predominant resistance mechanisms to the β-lactams in Gram-negative bacteria (Kaye and Pogue 2015; Bush and Bradford 2016; Chellat et al. 2016; Mo et al. 2018). In Figure 7 are represented the main mechanisms of resistance to β-lactams in Gram-negative pathogens in the Enterobacteriaceae family. The most common and damaging mechanism of β-lactam resistance, in these microorganisms, is bacterial hydrolases - β-lactamases (Kaye and Pogue 2015).

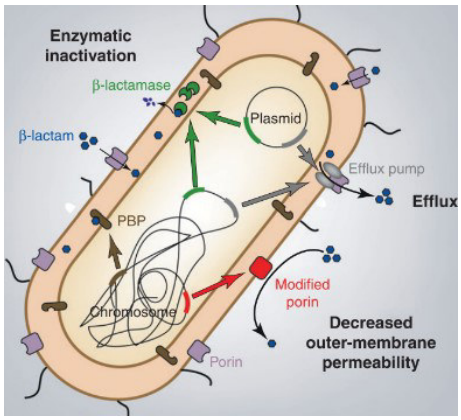


Figure 7 Mechanisms of β-lactam resistance in Enterobacteriaceae family (adapted from (Nordmann et al. 2012)).

1.4.3.1 β-lactamases

The term β-lactamase refers to the heterogeneous group of enzymes that hydrolyzes *β*-lactam molecules. These enzymes can be chromosomally encoded or plasmid-mediated. It may be produced at high levels and transferable on MGEs or by HGT, between different species (Bush and Bradford 2016; Bassetti et al. 2017; Moawad et al. 2018). There are different types of β-lactamases as referred to in section 1.3.1, and, they have a different amino acid composition and substrate profiles, so they can act against different β-lactams. Thus, it has been classified in two ways (El Salabi et al. 2013; Ghafourian et al. 2014):

The first classification is Ambler classification based on conserved and distinguishing amino acid motifs while Bush-Jacoby-Medeiros classification is based on functional characteristics of β-lactamases. According to Amber classification, β-lactamases are classified into four molecular classes, A (serine penicillinases), B (Metallo-β-lactamases (MBLs)), C (cephalosporinases), and D (oxacillinases). Classes A, C, and D include enzymes that hydrolyze their substrates by forming an acyl-enzyme through an active site serine, whereas class B are metalloenzymes which require divalent zinc ions to hydrolyze the substrate. Functional classification scheme takes into consideration the substrate and inhibitor profile of the enzymes, separating them into 4 groups (1-4) and several subgroups (a-f) (Brito and Lopes 1980; Bush et al. 1995; Bush and Jacoby 2010; El Salabi et al. 2013; Shaikh et al. 2015). These two classifications are described in Table 1.

Table 1 Classification of β-lactamases based on amino acid sequences and on functional characteristics (adapted from (Bush et al. 1995; Bush and Jacoby 2010; Kaye and Pogue 2015; Sheu et al. 2018)).

Amber class	Bush-Jacoby-Medeiros group	β-lactamases	Hydrolysis spectrum	Inhibited by CA/TZB	Representative enzyme(s)	Typical Organisms Producing these enzymes
A	2a	Penicilinases	Penicillins	Yes	PC1	*Staphylococcus aureus*
A	2b		Penicillins, narrow-spectrum cephalosporins	Yes	TEM-1, TEM-2, SHV-1	*Escherichia coli*, *Klebsiella* species, *Enterobacter* species
A	2be		Penicillins, extended-spectrum cephalosporins and monobactams	Yes	SHV-2 to SHV-9, TEM-3 to TEM-29, CTX-Ms	
A	2br		Penicillins	No	TEM-30 to TEM-40, SHV-10,26,49,56,72,84,107, TEM-50	
A	2c		Penicillins, carbenicillin	Yes	PSE-1, CARB-3	
A	2ce		Carbapenem, cefepime	Yes	RTG-4	
A	2e		Extended-spectrum cephalosporins	Yes	FEC-1, CepA	
A	2f		Carbapenems	Variable	KPC-2, SME-1, IMI-1, GES-2 to GES-17	
B (B1)	3a	Metallo- β-lactamases	Carbapenems	No*	IMP-1, VIM-1, NMD-1, CcrA, IND-1	*Klebsiella pneumoniae*, *E. coli*, *Klebsiella oxytoca*, *Enterobacter* species
B (B3)		Metallo- β-lactamases			L1, CAU-1, GOB-1, FEZ-1	
B (B2)	3b	Metallo- β-lactamases	Carbapenems	No*	CphA, Sfh-1	
C	1	Cephalosporinases	Cephalosporins	No	AmpC, CMY-2, ACT-1	*E. coli*, *Enterobacter* species, *Citrobacter* species, *Pseudomonas aeruginosa*, *Serratia* species
C	1c	Cephalosporinases	Cephalosporins	No	GC1, CMY-37	
D	2d	Oxacillinases	Cloxacillin	Variable	OXA-1, OXA-10	*Klebsiella pneumoniae*, *E. coli*, *Citrobacter freundii*, *Proteus mirabilis*, *Acinetobacter baumannii*
D	2de		Extended-spectrum cephalosporins	Variable	OXA-11, OXA-15	
D	2df		Carbapenems	Variable	OXA-23, OXA-48	

The first AmpC β-lactamase, with the capacity to hydrolyze penicillin, was discovered, in 1940, in *E. coli* strains (Ghafourian et al. 2014). Now, this enzyme is present in *Serratia marcescens*, *A. baumannii*, *Citrobacter freundii*, *Enterobacter cloacae* and other members of the Enterobacteriaceae family and it's also intrinsic to *P. aeruginosa* (Thabit et al. 2015; Khoshnood et al. 2017). AmpC genes in the chromosome produce a low-level

resistance but are inducible upon exposure to β-lactams, namely clavulanic acid, and imipenem, and thus become hyper-expressed.

Global dissemination of ESBL, mostly, CTX-M-15, mainly found in *E. coli* is one of the most significant examples of the rapid spread of resistance genes among bacterial pathogens (Sader et al. 2014a; Kaye and Pogue 2015; Shaikh et al. 2015). In Figure 8 is represented the number of β-lactamases identified between 1970 and 2015, where there is a drastic increase from 1995.

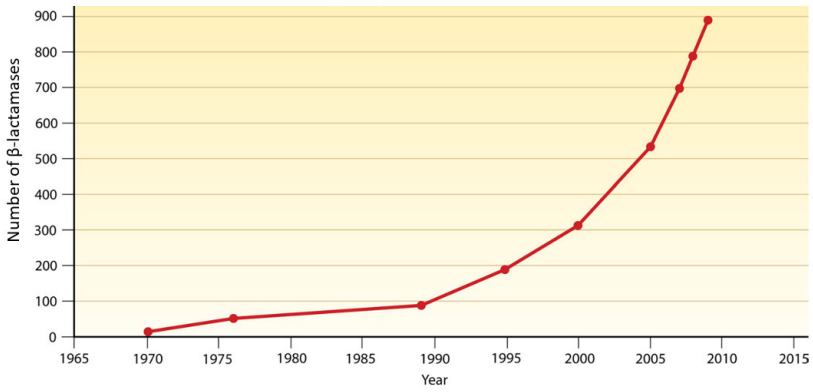


Figure 8 Numbers of β-lactamases enzymes identified since the introduction of the first β-lactam antibiotics (adapted from (Davies and Davies 2010)).

In critically ill patients, with infections caused by ESBL-producing such as *E. coli* and *Klebsiella* spp., carbapenems are considered first-line therapy, since these bacteria are resistant to nearly all penicillins and third generation cephalosporins (Shaikh et al. 2015; Morehead and Scarbrough 2018). However due to their excessive use, there are a huge spread of Carbapenemase-producing Enterobacteriaceae (CPE) which have a broad spectrum of hydrolytic activity preventing its action (Martirosov and Lodise 2016; Giacobbe et al. 2018; Sultan et al. 2018).

Carbapenemases and ESBL genes are usually located in plasmids or other MGEs and it can be transferred horizontally between species, so there is a high potential for its dissemination. This allows organisms to acquire genes that confer resistance to different antimicrobial classes, giving rise to XDR or even PDR pathogens, especially *E. coli*, *Klebsiella* spp., *Pseudomonas* spp., and *Acinetobacter* spp. (Khoshnood et al. 2017; Theuretzbacher 2017; Alfouzan et al. 2018; Moawad et al. 2018).

1.4.4 Ceftolozane/Tazobactam (Cef/taz)

The development of new antibiotic agents would greatly assist in controlling the most prevalent infectious diseases. As a potential alternative to carbapenems, new antimicrobials, resistant to inactivation by ESBLs, are under development.

Ceftolozane, previously known as CXA-101 and FR264205, is a novel broad-spectrum cephalosporin with a structural change in relation to ceftazidime. This change prevents it from being hydrolyzed by β-lactamases, namely AmpC β-lactamases (van Duin and Bonomo 2016; Bradley et al. 2018; Giacobbe et al. 2018).

Tazobactam is a penicillinate sulfone β-lactamases inhibitor, which protects ceftolozane the hydrolysis of the β-lactam ring, once it can bind and inhibit the most common class A and C β-lactamases, by binding to the active site of these enzymes (Cho et al. 2015; Saran et al. 2019).

Ceftolozane/tazobactam (Cef/taz) is a β-lactam/β-lactamases inhibitor combination both chemical structures are shown in Figure 9.

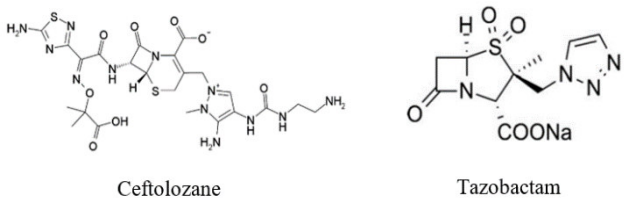


Figure 9 Chemical structure of ceftolozane and tazobactam (adapted from (Cho et al. 2015)).

This combination antimicrobial has been developed for clinical use because tazobactam increases the activity of ceftolozane against most ESBL-producing Enterobacteriaceae including TEM, SHV, and CTX-M types and some Bacteroides spp. (Giacobbe et al. 2018; Saran et al. 2019). This new antimicrobial agent was developed to address the rising rates of antimicrobial resistance in Gram-negative pathogens and as all β-lactams, their bactericidal activity consists of inhibit bacterial cell wall biosynthesis (Cho et al. 2015; Saran et al. 2019).

1.4.4.1 Spectrum of activity

Cef/taz is the most active β-lactam against Gram-negative pathogens ESBL-producing such as *E. coli*, *K. pneumoniae,* and *P. mirabilis.* For these microorganisms, Cef/taz demonstrated overall activity superior to that of piperacillin/tazobactam, and then

available cephalosporins, and higher against *E. coli* than against *K pneumoniae* (Cluck et al. 2015; Melchers et al. 2015; Giacobbe et al. 2018). This compound is also active against *P. aeruginosa*, *Haemophilus* spp., *Moraxella* spp., and also against some strains of *Acinetobacter*, *Stenotrophomonas*, *Burkholderia* and other nonfastidious Gram-negative nonfermenters (Giacobbe et al. 2018). The activity against anaerobic and Gram-positive organisms is limited like many cephalosporins, it's mainly used against Streptococcus and Staphylococcus species (Cho et al. 2015; Montravers and Bassetti 2018).

This compound has a higher affinity and a broader inhibition profile toward the essential PBPs of *P. aeruginosa*, it's particularly potent for PBP3, while the affinity to PBP4 remains lower than that of imipenem and thus unable to induce AmpC overexpression. Other reason to high efficacy of Cef/taz to this strain, unlike carbapenems, is that it has better outer membrane permeability so its' entry across the outer membrane is not affected by functionality of the OprD porin (Cho et al. 2015; MacVane et al. 2017; Giacobbe et al. 2018).

The treatment of XDR *P. aeruginosa* infections is very limited, there are a few available therapeutic options because of the complexity of these infections in patients with high comorbidity. Another problem for this microorganisms is the need for combination therapy (Escolà-Vergé et al. 2018). Thus, the great advantage of Cef/taz is its' lower propensity for resistance development in comparison with other antipseudomonal agents *in vitro*, thank to its' higher stability against *P. aeruginosa* mutational resistance mechanisms (Díaz-cañestro et al. 2018).

In general, CXA-101 profile against Gram-positive and Gram-negative pathogens is similar to that of third-generation cephalosporins, such as ceftazidime, but its antipseudomonal activity is the most potent among all currently available β-lactams, including carbapenems (Ge et al. 2010).

1.4.4.2 Mechanisms of resistance

Cef/taz activity is prevented by the hydrolysis of carbapenemases such as MBLs, *K. pneumoniae* carbapenemase (KPC) and the most Class D β-lactamase-producing organisms (Giacobbe et al. 2018; Hawkey et al. 2018; Montravers and Bassetti 2018). Furthermore, de-repressed/overexpression, possibly resulting from a mutation, in the resident AmpC β-lactamase in strains like *Acinetobacter* spp. and *Enterobacter* spp. or structural changes in *P. aeruginosa* also retain the activity of Cef/taz (Cluck et al. 2015; Livermore et al. 2017; Díaz-

cañestro et al. 2018; Escolà-Vergé et al. 2018). However, this antimicrobial agent maintains susceptibility to other forms of resistance such as efflux pumps and loss of porin channels (Cluck et al. 2015; van Duin and Bonomo 2016).

Dissemination of resistance to most available antibiotics such as β-lactams, fluoroquinolones and aminoglycosides is increasing worldwide. CPE, ESBL-producing *E. coli* and *K. pneumoniae, A. baumannii*, and *P. aeruginosa* are increasingly prevalent in infectious diseases (Bialvaei and Samadi Kafil 2015; Dandan et al. 2019). Due to the emergence of these infections and the current paucity of novel antibiotics to combat them, scientists were forced to resort to "old" antibiotics, mainly colistin (polymyxin E), polymyxin B, fosfomycin and tigecycline because there is no other less toxic and at least as effective (Shaikh et al. 2015; Jeannot et al. 2017; Poirel et al. 2017; Rabanal and Cajal 2017; Alfouzan et al. 2018).

Thus, currently, these antibiotics are considered the last line of treatment options for patients with infectious diseases caused by difficult to treat microorganisms, chiefly CPE.

1.4.5 Polymyxins

The polymyxin family is a group of antimicrobial cyclic lipopeptides produced by the fermentation of strains of *Bacillus polymyxa* that are divided into five different chemical compounds (polymyxins A, B, C, D, and E), with multiple components (Jeannot et al. 2017; Poirel et al. 2017; Rabanal and Cajal 2017).

Colistin (polymyxin E) and polymyxin B (chemical structure, shown in Figure 10) are the most common members of this family; they were used in veterinary medicine approximately from the late 1950s to the late 1970s. They have been used for prophylactic of infectious disease, manly enterobacterial infections caused by *E. coli* in poultry and pigs and as a growth factor in animal production. In 2011, these class of antibiotics was the fifth most sold (7%) for treating food-producing animals in Europe (Poirel et al. 2017; Forde et al. 2018). This compounds were extensively used owing to its ability to bacterial killing, narrow spectrum of activity, and until now, slow development of resistance (Bialvaei and Samadi Kafil 2015). However, its use is associated with some toxicity, which is dose-dependent and reversible when the treatment ends. The main disadvantages of using colistin are renal and neurological levels. Escolà-Vergé et al. 2018 concluded that up to 44% of patients treated with polymyxins, rather colistin developed acute kidney injury. The rate of

colistin-associated neurotoxicity is approximately 7%. Thanks to this, these antimicrobial agents were gradually substituted by other antibiotics in human therapy, but in veterinary medicine, it has always continued to be used (Rabanal and Cajal 2017; Rhouma and Letellier 2017).

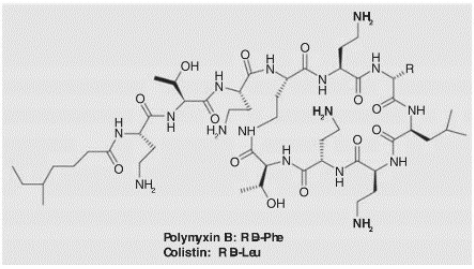


Figure 10 Polymyxin B and Colistin (polymyxin E) (adapted from (Biswas et al. 2012)).

1.4.5.1 Spectrum of activity

Polymyxins are active against Gram-negative bacteria such as *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Salmonella* spp., and *Shigella* spp and for common nonfermentative Gram-negative bacteria, including *A. baumannii* and *P. aeruginosa*. However, polymyxins have no activity in Gram-negative cocci, Gram-positive and anaerobic bacteria. Some microorganisms like *Proteus* spp., *M. morganii*, *Providencia* spp., *S. marcescens*, *Pseudomonas mallei*, *Burkholderia cepacia*, *Chromobacterium* spp., *Brucella*, *Legionella*, *Campylobacter*, and *Vibrio cholerae* have mechanisms of intrinsic resistance, so they are naturally resistant to polymyxins (Biswas et al. 2012; Bialvaei and Samadi Kafil 2015; Liu et al. 2016; Poirel et al. 2017; Rabanal and Cajal 2017).

1.4.5.2 Mechanism of action

Polymyxins act essentially on Gram-negative bacteria, since their mechanism of action is the direct interaction with lipopolysaccharide (LPS), which is the main constituent of the outer membrane of these bacteria. When the LPS is destabilized, the permeability of the bacterial membrane increases, leading to a higher uptake of the drug (autopromoted absorption) and, consequently, the leakage of the cytoplasmic content, culminating in cell death (Bialvaei and Samadi Kafil 2015). Figure 11 schematically represents the mechanism of action of colistin.

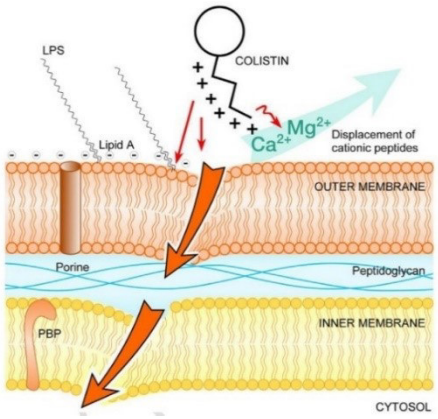


Figure 11 Mechanism of action of colistin in Gram-negative bacteria (adapted from (Bialvaei and Samadi Kafil 2015)).

Colistin has become the last resource active against emerging MDR bacteria. Unfortunately, its inappropriate and increased use led to colistin resistance selection, especially in CPE-endemic countries (Baron et al. 2016; Poirel et al. 2017; Jousset et al. 2018).

1.4.5.3 Mechanisms of resistance

The emergence of polymyxins resistance, in some countries, has been considered as a huge threat to public health (Bialvaei and Samadi Kafil 2015; Kaye and Pogue 2015). Resistance to polymyxins can be intrinsic or acquired. As already mentioned, there are some species that present intrinsic resistance to colistin, without having been exposed to it. Probably, this species developed spontaneous mutations of genomic DNA, which are inherited, low-level, and independent of the continuous presence of the antibiotic (Cheng et al. 2015).

In Gram-negative bacteria, the acquisition of resistance can be by several molecular mechanisms, such as specific modification of outer membrane porins, overexpression of efflux pump systems, overproduction of capsule polysaccharide and even colistinase producing species have been identified (*B. polymyxa)* (Bialvaei and Samadi Kafil 2015). The most common mechanisms of resistance in members of the Enterobacteriaceae family like *K. pneumoniae*, *E. aerogenes*, *S. enterica* and more recently in *E. coli* are mediated by chromosomal mutations as also happens in *P. aeruginosa* and *A. baumannii* (Baron et al. 2016; Poirel et al. 2017; Bardet and Rolain 2018). This mechanism results mostly in modifications of the main target of polymyxins - LPS (Forde et al. 2018). These

modifications occur through the addition of cationic groups through mutations on genes and operons encoding proteins that are involved in the bacterial cell (Jeannot et al. 2017; Jousset et al. 2018). These cationic groups decrease the electronegative charge of the LPS leading to electrostatic repulsion of the positively charged polymyxin. These loads change reducing the affinity of polymyxin molecules, preventing its' binding and subsequent entry into the bacterial cell (Liu et al. 2016; Poirel et al. 2017).

1.4.5.3.1 The *mcr*-1 gene

Initially, the transmissible, plasmid-borne colistin resistance *mcr-1* gene was found in animals, particularly pigs and cattle (Zurfluh et al. 2017; Cao et al. 2018; Moawad et al. 2018). This may have been the result of the high use of polymyxins in veterinary medicine as a growth promoter, prophylaxis and metaphylaxis, mostly in pigs, chickens and cattle (Nordmann and Poirel 2016; Poirel et al. 2017). In 2015, when the *mcr-1* gene was discovered in Southern China, a re-evaluation was made in the use of colistin for veterinary medicine and as a feed additive. However, despite extensive use of colistin, the resistance rate from healthy animals remains < 1% in many European countries (Sun et al. 2018).

The *mcr-1* gene encodes for a phosphoethanolamine transferase, it has the capacity to transfer cationic groups to LPS, altering it. This enzyme confer resistance to colistin using the same mechanisms which has already been studied in intrinsically resistant Gram-negative species. Without additional resistance mechanisms, production of MCR-1 enzyme is enough to confer resistance to colistin (Poirel et al. 2017; Bardet and Rolain 2018).

Until today, eight types of *mcr* genes have already been described: *mcr-1* (1626 bp), *mcr-2* (1617 bp), *mcr-3* (1626 bp), *mcr-4* (1626 bp), *mcr-5* (1644 bp), *mcr-6* (1617 bp), *mcr-7* (1620 bp), and *mcr-8* (1698 bp); The *mcr-1* gene already has 12 variants (*mcr-1.2*, *mcr-1.3*…*mcr-1.12*) that have been reported in Enterobacteriaceae members. More than 80% were found in *E. coli,* but some epidemiological studies have detected *mcr*-1 gene also in *K. pneumoniae*, *Enterobacter* spp, *S. enterica*, *Shigella sonnei* and *Citrobacter* spp., (Jeannot et al. 2017; Li et al. 2018; Sun et al. 2018; Yang et al. 2018). *Mcr* genes become a major global health problem because they are located on transferable plasmids, which can easily propagate by conjugation among *E. coli* strains and by *in vitro* transformation to *K. pneumoniae* and *P. aeruginosa* (Poirel et al. 2017; Forde et al. 2018).

Mcr-2 to *mcr-5* genes are more prevalent in animals than human isolates. In human isolates the most common is *mcr-1* gene, but still, its rate remains low (≤ 2% of clinical Enterobacteriaceae isolates in China and ≤ 0.2% of clinical *E. coli* isolates in Europe) due to the fact that in hospitals the use of colistin is still low (Forde et al. 2018; Jousset et al. 2018). Nowadays, due to the increase of the use of this antibiotic to treat infections caused by MDR Gram-negative bacteria, the colistin resistance begins to spread in several countries worldwide, even in countries where colistin isn't used (Bialvaei and Samadi Kafil 2015; Butaye and Wang 2018). This gene has already been detected from over 25 countries throughout Asia, Europe, the Middle East, North Africa and America (Jeannot et al. 2017; Poirel et al. 2017; Forde et al. 2018; Wyres and Holt 2018). The high variety of *mcr-1*-bearing plasmids detected in members of the Enterobacteriaceae family from different continents confirm their capacity to globally spread. Figure 12 shows the globally spread of the *mcr-1* gene from different sources.

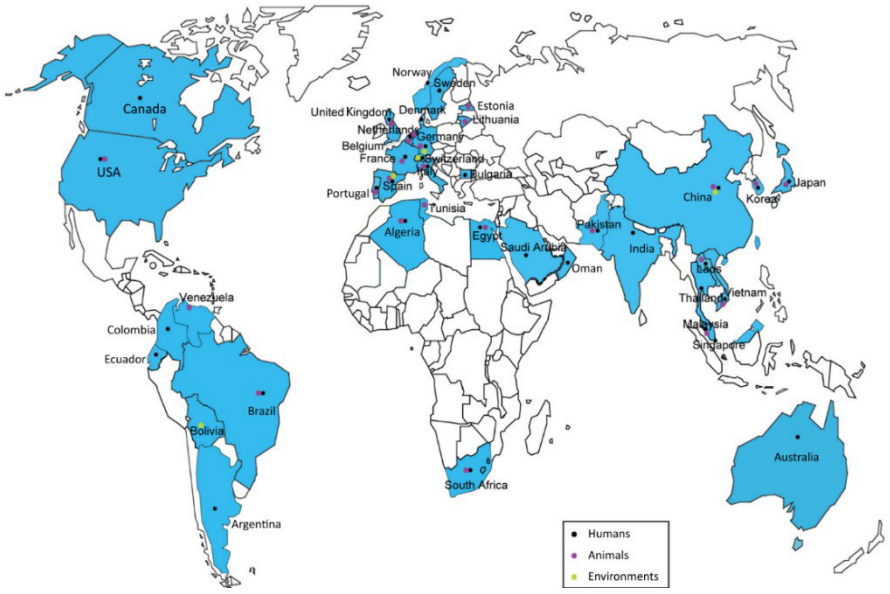


Figure 12 The *mcr-1* gene human, animals and environments isolates (adapted from Poirel et al. 2017)).

Some surveillance studies reported colistin resistance in *K. pneumoniae* in many European countries, but with very different rates. Romania (25.8%), Greece (19.9%) and Italy (15.4%) are the countries with the highest rates, according to the global SENTRY Antimicrobial Surveillance Studies. The European Center for Disease Prevention and Control conclude that polymyxin resistance in *K. pneumoniae* can be more critical than in *E. coli*, with rates very high in Europe (8.2% in 2014) (Poirel et al. 2017).

1.4.5.4 Co-occurrence of ESBLs, carbapenemases and *mrc-1* gene

Plasmid-mediated *mcr-1* gene is frequently associated with the carriage of other antimicrobial resistance genes, namely ESBL and carbapenemases like bla_{NDM-1}, bla_{NDM-5}, bla_{NDM-9}, bla_{OXA-48}, bla_{KPC-2} and bla_{VIM} (Baron et al. 2016; Zurfluh et al. 2017; Cao et al. 2018; Moawad et al. 2018). The first report of resistance was by ESBL-producing microorganisms and at the temporal level, this coincided with the development of the *mcr-1* gene in *E. coli* (Rhouma and Letellier 2017). Findings of Haenni et al. 2016 demonstrate that there is a co-location of the *mcr-1* gene and ESBLs, on a single plasmid but additional studies are needed to clarify the diversity of the plasmid backbones. Figure 13 indicates the emergence of colistin resistance as well as ESBLs and carbapenemases genes.

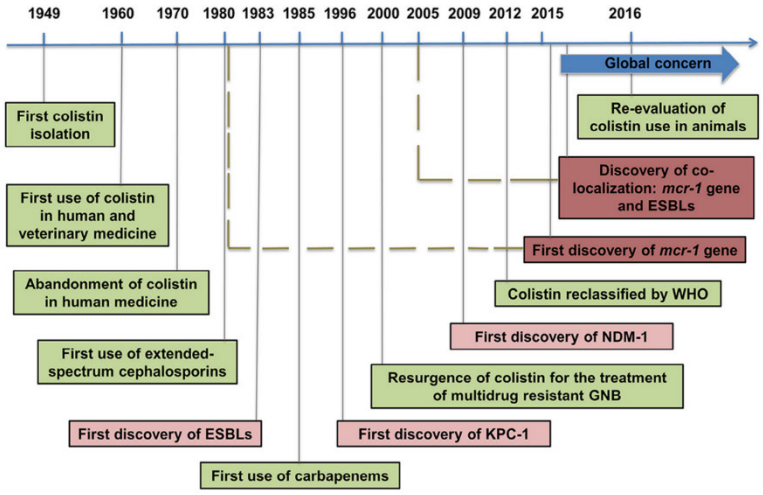


Figure 13 Schematic representation of historical dates about identification of ESBLs, carbapenemases enzymes and *mcr-1* gene (adapted from (Rhouma and Letellier 2017)).

The genes encoding ESBLs or carbapenemases alter the chromosomal *mgrB* gene which is an important gene related with colistin resistance. When these genes are both present, the isolates can be resistant to β-lactams, aminoglycosides, quinolones, fosfomycin, sulfonamides, and tetracyclines which are the antimicrobial agents clinically relevant for human medicine (Poirel et al. 2017). This phenomenon is very worrying since these plasmids have been found in highly drug resistant Enterobacteriaceae isolates and it could be the cause of the emergence of pan drug resistance in the Enterobacteriaceae (Haenni et al. 2016; Forde et al. 2018; Jousset et al. 2018).

Strains harboring mutations in the different genes (phoPQ, pmrAB, *mgrB*, and *mcr-1*) suggests a strain-specific pathway, that is, heteroresistance to polymyxins. This is defined as a heterogenous response of bacterial cells within the same population to a given antibiotic. Heteroresistant strains such as *K. pneumoniae*, *A. baumannii* , and *P. aeruginosa* originates subpopulations with a wide range of susceptibility levels to polymyxins (Jeannot et al. 2017; Rabanal and Cajal 2017; Ezadi and Ardebili 2018). This may be a threat to the effectiveness of diagnostic tests and patient treatment because is difficult to evaluate the correct MIC value. Heteroresistant is mostly observed with monotherapy, which is related to the rapid emergence of colistin resistance. To try to combat the great and rapid spread of resistance, currently, it is chosen the antibacterial combination therapy (Bialvaei and Samadi Kafil 2015; Rabanal and Cajal 2017).

1.4.5.5 Antibacterial combination therapy with colistin

Carbapenem-resistant Enterobacteriaceae are the most worrisome microorganisms for which the combination of colistin with other antibiotics appears to be the only remaining option. As polymyxins affect the integrity permeability of the outer membrane of Gram-negative bacteria, they can help to increase activity and accumulation of other antibiotic classes into bacterial cells (Biswas et al. 2012; Rabanal and Cajal 2017). The synergistic effect against Gram-negative bacteria, particularly *P. aeruginosa*, *K. pneumoniae* and *A. baumannii*, maximize the efficacy of polymyxins decreasing the emergence of resistance and, at the same time, it can reduce the toxicity of these drugs. Thus, the most common antibiotics used for synergistic effect with polymyxins, mainly colistin, are carbapenems followed by rifampicin, tigecycline, imipenem, arbekacin, ceftazidime, aztreonam, piperacillin, amikacin, fosfomycin, gramicidin, glycopeptides and ciprofloxacina. Rifampicin has now been shown to be the best option for the treatment of MDR and XDR Gram-negative bacterial infections (Bialvaei and Samadi Kafil 2015; Liu et al. 2016; Rabanal and Cajal 2017).

Combination therapy confers a higher microbiological eradication rates, and a low in-hospital mortality rate compared to colistin monotherapy (Bialvaei and Samadi Kafil 2015).

1.5 Impact of antimicrobial resistance in society

In Europe, it is estimated that by 2050, mortality attributed to antibiotic resistance will be associated with approximately 10 million deaths annually (WHO 2014; Potron et al. 2015; Marston et al. 2016; Montravers and Bassetti 2018; Koulenti et al. 2019). The new plasmid-mediated genes that confer resistance to colistin also contributes to an increase in this already worrying data (Bassetti et al. 2017). The costs associated with antimicrobial resistance were expected to be over $105 billion annually worldwide (Codjoe and Donkor 2017). The severity of this problem is different between countries, being more severe in Asia, southeast Europe, South America, and Africa. In Figure 14 is represented the expected impact of antimicrobial resistance in 2050 in different countries (Bassetti et al. 2017).

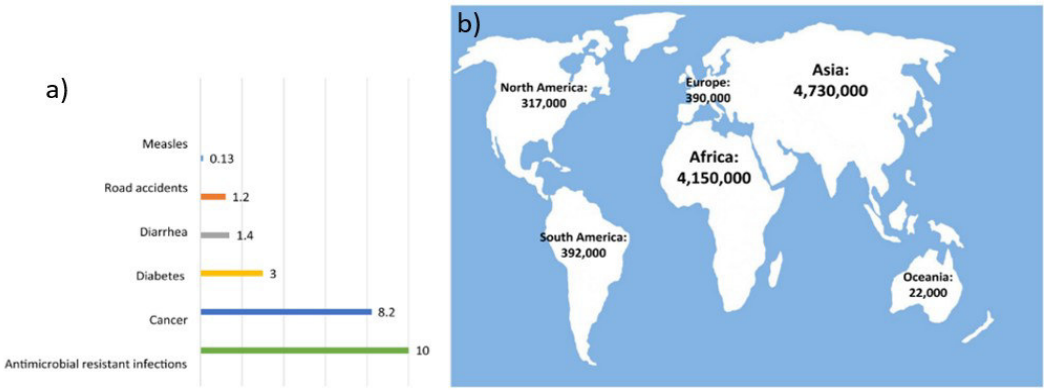


Figure 14 The impact of antimicrobial resistance in 2050. a) deaths per year by antimicrobial resistant infections and other causes in millions; b) death associated to antimicrobial resistance every year by 2050 in different countries (adapted from (Bassetti et al. 2017)).

The causes leading to increased antimicrobial resistance differ between developed and developing countries. In the developed countries, the principal cause is indiscriminate prescribing of antibiotics due to patient expectations and uncertain diagnoses while in the developing world, it's the unregulated availability of antibiotics in the community, which leads to ill-advised self-medication. Self-medication, normally, is fraught with inadequate dosing and duration of use as well as unnecessary consumption (Morehead and Scarbrough 2018).

Antimicrobial resistance genes are derived from environmental bacteria, particularly of soil, which have coevolved with antimicrobial producing organisms for long periods of time (Wyres and Holt 2018). The misuse of antibiotics by food and animal industries for animal treatment and, more significantly for infection prevention and growth promotion, also

have contributed to the increase of antibiotic resistance (Kaye and Pogue 2015; Marston et al. 2016; Morehead and Scarbrough 2018). Other environmental factors that cause this phenomenon are the wastes from large farms, municipal wastewater containing partially metabolized or discarded medications, industrial agricultural plants with water and ground dissemination of drugs, and naturally occurring pathogenic bacteria found in waterways and soil. In Figure 15 are shown some factors that contribute to the dissemination of antibiotic resistance and how they are related to each other.

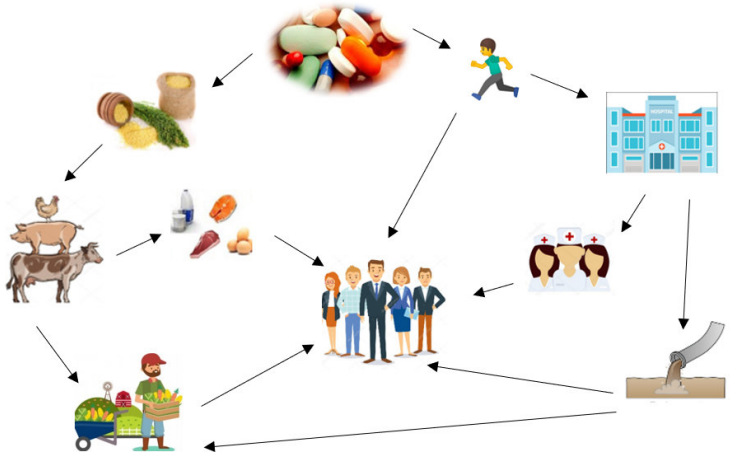


Figure 15 Dissemination of antibiotic resistance among environment, animals, hospital, and community (adapted from (Davies and Davies 2010)).

E. coli and *Klebsiella* spp. are two pathogens of interest since they have a highly fluid genome, mediated by MGEs that contain antibiotic resistance and pathogenicity genes that allow the propagation of resistance from environment to animals and humans (Bouxom et al. 2018; Morehead and Scarbrough 2018; Wyres and Holt 2018). Figure 16 is a schematic representation of the dissemination of resistant bacteria between environment, animals, and humans.

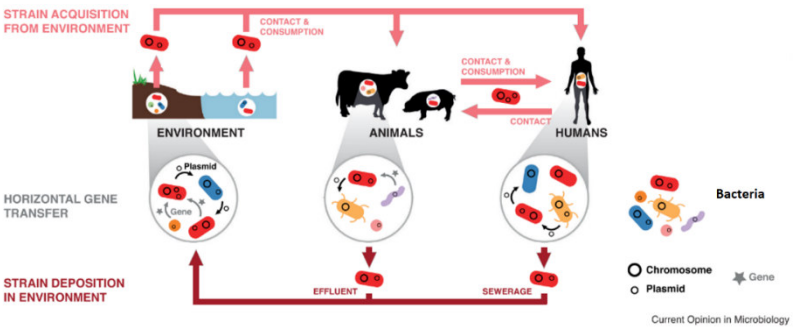


Figure 16 Dissemination of resistant bacteria between environment, animals and humans (adapted from (Wyres and Holt 2018)).

The growing emergence of MDR and XDR strains and the global dissemination of ESBLs, as well as carbapenemases, has increased the likelihood of empirical therapy, or it will be ineffective in many cases, increasing mortality (Hawkey et al. 2018; Montravers and Bassetti 2018; Koulenti et al. 2019). It has been estimated that infections caused by microorganisms producing these enzymes, mainly *E. coli* and *Klebsiella* spp. are associated with an increased risk of death and costs 3.6-fold and 1.7-fold more, respectively, compared with strains without these resistant mechanisms (Kaye and Pogue 2015).

Advanced age, underlying renal or liver pathology, nursing home residence, catheterization or use of nasogastric tubes, prolonged hospitalization, and previous antibiotic treatment have been proposed as risk factors in infections caused by ESBLs producer strains (Medina-Polo et al. 2015; Shaikh et al. 2015). Patients with these infections may be subjected to a delay in the identification of infection as well as in an initial appropriated antimicrobial therapy (Ghafourian et al. 2014; Thabit et al. 2015). In these cases, normally, extended spectrum antibiotics are used, because these ensure adequate treatment of many infections, but, at the same time, it's a major argument for antibiotic overuse in the hospital setting and is resulting in continuous pressure towards the selection of resistance. Resistance to many different types of drugs such as cephalosporins, aminoglycosides, fluoroquinolones and the emerging resistance to polymyxins, limit the choices for definitive therapy, mainly to community infections or hospital-acquired infections (Shaikh et al. 2015; Chellat et al. 2016; Marston et al. 2016; Bassetti et al. 2017).

Access to clean water and sanitation and hospital infection control can prevent bacterial infections and consequently decrease the use antibiotics but, the key to solving the emergence of resistant mechanisms is the development of new antibiotics (Kanj and Kanafani 2011; Marston et al. 2016; Alfouzan et al. 2018). However, the development of new antibiotic has been decreasing since the 1980s, and decreased drastically after the 2000s (Shaikh et al. 2015; Chellat et al. 2016; Bassetti et al. 2017). Since the first carbapenem became available in 1985, no antibiotic with a new chemical structure, an unexplored target or a new mode of action has yet been developed (Theuretzbacher 2017). Figure 17 shows some important events in the age of antibiotics.

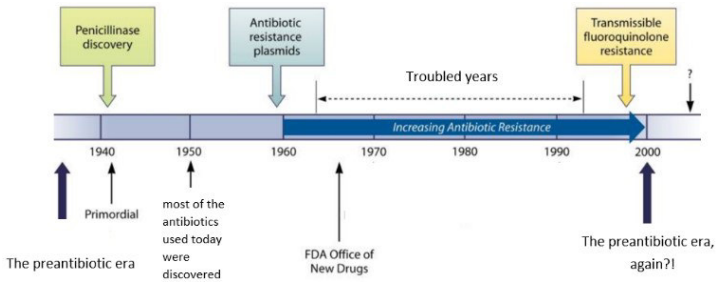


Figure 17 Development of antibiotics and concomitant resistance (adapted from (Davies and Davies 2010)).

In many countries, resistance rates are still low but, in countries such as Greece and Italy, it's getting higher and higher. In addition, the recent identification of *mcr-1* gene in human, animal and environmental strains of Enterobacteriaceae indicates that the current situation may further worsen at the global scale (Jeannot et al. 2017; Poirel et al. 2017). Colistin may be the last antibiotic agent for treatment of MDR Gram-negative bacteria, especially when these bacteria are resistant to carbapenems. The dissemination of *mcr-1* gene among these bacteria is very problematic and may herald the postantibiotic era. It's extremely necessary to develop an antimicrobial-resistant containment strategy to try to reduce the spread and transmission routes of *mcr-1* carrying bacteria as well as other resistance genes (Haenni et al. 2016; Paveenkittiporn et al. 2017).

2 Scope

The global spread of resistant strains to most clinically available antibiotics is increasing, especially in the hospital setting. Based on this worrisome public health problem, this main goal of this thesis is to contribute to the knowledge of distribution and prevalence of resistance in selected species collected from biologic products of inpatients and outpatients in the "Centro Hospitalar do Baixo Vouga, E.P.E., Portugal" (CHBV). Thus, this is organized into three distinct chapters.

a) Chapter 1: It is intended to perform the surveillance of *mcr-1* gene dispersion in the CHBV area of influence. To do so, all microorganisms, from 2017 to May 2019, whose antimicrobial susceptibility test (AST) result was resistant to the antibiotic colistin were collected, from in- and outpatients, regardless the diagnosis, biological product or the hospital ward from which they were retrieved.
b) Chapter 2: *In vitro*, to evaluate, the efficacy of Cef/taz strips against β-lactamases-producing Enterobacteriaceae strains and *Pseudomonas aeruginosa* isolated from in- and outpatients of CHBV with suspected UTI, from September to November 2018.
c) Chapter 3: To develop pedagogical materials for the community awareness of the problem mentioned above, through participation in a pilot project – "Projeto de Estímulo à Mobilidade de Ideias" (PEMI) - that is one of the educational projects developed by the Association for World Innovation in Science and Health Education (AWISHE) Infection Control Awareness Through Education (ICATE).

3 Material and methods

3.1 Central Hospital characterization

The present study was performed in the section of Microbiology of the Clinical Pathology Department, in the CHBV. This hospital includes several wards, namely internal medicine, general surgery, orthopedics, pediatrics, urology, infectiology, cardiology, pneumology, gynecology and obstetrics. The ER of this hospital also comprises several medical specialties, in which clinical pathology is included.

Urine, sputum, pus, and blood samples are among the main biological products sent for microbiological analysis.

All the isolates analyzed in this study were collected from inpatients and outpatients attending the CHBV, in a selected timeframe, from 2017 to May 2019.

3.2 Clinical data collection

The clinical data including specimen type, date of hospital admission, age, gender, hospital ward, infection type, previous infections, subsequent therapies, and clinical outcomes were collected from patients with strains analyzed in this study. Clinical history of these patients was analyzed retrospectively.

3.3 Bacterial strains

In the Microbiology Laboratory, the different samples were cultured in the adequate culture medium, selected according to the standards of the microbiology section. All urine samples were inoculated in CLED (Cystein Lactose Electrolyte Deficient medium) (BioMérieux, France). Other types of biologic products, like blood, sputum or pus, were inoculated in another culture medium. If microorganisms were detected in the culture plates, colonies were inoculated to MacConkey Agar (BioMérieux, France), which is a selective and differential medium, Columbia Agar (BioMérieux, France), with 5% sheep blood or PolyViteX Chocolate Agar (BioMérieux, France). All plates were then incubated for 16-24 hours at 37/35 ºC. Urine samples, in CLED (Figure 18), with a single strain were considered significant bacteriuria if the culture had $1x10^5$ CFU/mL. The microorganism in question was then identified and an antibiotic panel tested.

Figure 18 Sample of urine inoculated in a culture plate of CLED medium.

3.4 Gram staining

Bacilli or coccus differentiation was performed using Gram staining. This method distinguishes between Gram-negative and Gram-positive bacteria, based on differential staining with a crystal violet-iodine complex and a safranin counterstain.

Gram staining and biochemical tests were also made to help select the identification card to be used in the automated broth microdilution method Vitek2® (BioMèrieux, France).

3.5 Identification and susceptibility testing

All the isolates included in this study were non-duplicate and identified with the automated broth microdilution method Vitek2® (BioMèrieux, France), in accordance with EUCAST guidelines (version 9.0, 2019).

To identify all microorganisms, there are four distinct identification cards available: Gram-negative (GN/AST- N355) and Gram-positive bacteria, anaerobes and fungi. All cards contain a control well, where there is only culture medium. Each card used contains a bar code, reporting the type of ID card, lot number, expiring date and the corresponding sample identification for the equipment (Figure 19). The inoculum previously performed from a pure and fresh culture, used for the identification of the microorganism is used to perform the AST.

Figure 19 ID cards.

To assess antimicrobial susceptibility, the pure and fresh culture is taken to a suspension of 0,55 - 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. Figure 20 shows the automated broth microdilution method Vitek2® (BioMèrieux, France), present in the CHBV.

Figure 20 Automated broth microdilution method Vitek2® (BioMèrieux, France) present in the CHBV (https://www.biomerieux-usa.com/clinical/vitek-2-healthcare).

Each card has 64 microwells with a selected battery of antibiotics in different concentrations. The instrument monitors each of the wells, and 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). Quality control was performed using reference strains *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853, the results are only acceptable when the equipment is verified by the quality control system.

3.6 Cryopreservation

Strains that match the criteria selected were collected for cryopreservation tubes (CRYOBANKTM MIXED) with 2 mL, each containing 1 mL of hypertonic cryopreservative solution covering approximately 25 glass beads to which microorganisms can adhere. Colonies from a fresh and pure culture were aseptically inoculated in the CRYOBANK tubes that are stored in a suitable freezer at -20 ºC. When these microorganisms are required to perform the various tests, they are thawed and subsequently withdrawn from the tubes with sterile forceps into a suitable culture medium. At the end, if these strains are necessary for further studies, the tubes should be closed and placed as soon as possible on the freezer.

3.7 Phenotypic methods

3.7.1 Quantitative method - Liofilchem Ceftolozane-tazobactam MIC Test Strip (MTS)

A fresh and pure culture was used to achieve 0,5 McFarland standard turbidity suspension in a NaCl solution (DENSIMAT, BioMérieux) and was than spread into a Mueller Hinton Agar plate, in all directions, with a sterile swab, as shown in Figure 21. Test strips with predefined gradient of concentration of Cef/taz, that range between 0.016/4 - 256/4 µg/mL, were placed on the agar surface with the scale facing upwards, pressing it with sterile forceps. After that, the plates were incubated in an inverted position for 24 hours, at 37 ºC and the ellipse formed in each plate was interpreted. The MIC result was considered as the interception of the ellipse inhibition zone with the strip; if the ellipse intersected between two MIC values (Figure 21), the higher of the two values was reported. The procedures and criteria for interpretation – sensitive, intermediate and resistant - established by FDA were followed. FDA breakpoints for *P. aeruginosa* are Sensitive ≤ 4/4 and Resistant ≥ 16/4 µg/mL. For Enterobacteriaceae members, values are different: it's Sensitive ≤ 2/4 and Resistant ≥ 8/4 µg/mL. The strips and the results were used and interpreted according to the manufactures instructions. All materials needed are represented in Figure 22.

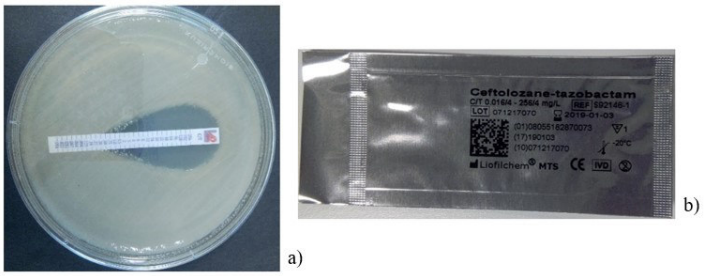


Figure 21 a) ellipses formed after 24h incubation; b) strips Cef/taz;

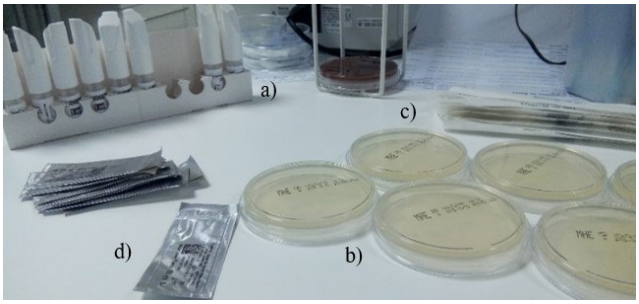


Figure 22 a) NaCl solution; b) Mueller Hinton Agar plate; c) sterile swab; d) strip Cef/taz.

3.7.2 Quantitative method - MICRONAUT-S broth microdilution Colistin MIC test (Merlin Diagnostika)

A fresh and pure culture was used to achieve 0,5 McFarland standard turbidity suspension in a NaCl solution (DENSIMAT, BioMérieux). 50 µL of this 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 (the first well) and an increasing concentration of colistin in the remaining wells, as demonstrated in Figure 23. Finally, the plate must be sealed with the unperforated plate sealer and incubated at 35-37 °C during 18-24 hours. The results were interpreted visually, according to the manufactures instructions, being the MIC value reported as that where bacterial growth did not occur.

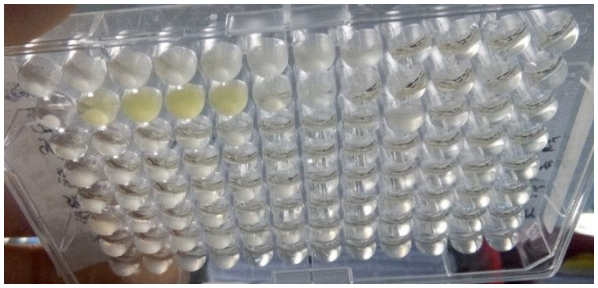


Figure 23 MICRONAUT-S broth microdilution Colistin MIC test (Merlin Diagnostika) plate with wells with a colistin concentration gradient.

3.7.3 Qualitative method – NG-Test MCR-1 (NG Biotech, France)

A bacterial colony obtained by a fresh and pure culture from agar plate was suspended in 150 µL extraction buffer provided into the kit (extraction step). After vortexing the microtubes to homogenize the mixture, 100 µL of the prepared mixture were loaded on the well labeled "S" in the cassette. The results were interpreted visually as positive when, after 15 min, two red lines appeared, one on the control region and one on the test region. The entire protocol is schematically represented in Figure 24.

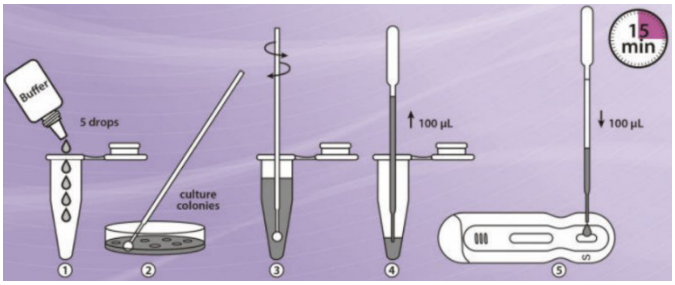


Figure 24 Schematic representation of the protocol for NG-Test MCR-1 (NG Biotech, France) (adapted from (https://ngbiotech.com/antibiotic-resistance/)).

3.8 Genotypic method

3.8.1 Polymerase Chain Reaction (PCR) amplifications

To extract the DNA template for further PCR amplification, from the intended strains, a bacterial colony of a fresh and pure culture was suspended in 50 µL of distilled water, as demonstrated in the Figure 25.

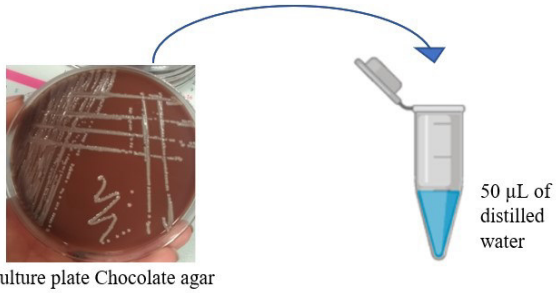


Figure 25 Schematic representation of the procedure for extracting the DNA template.

Box-PCR analysis with a single primer BOX A1R (5'-CTACGGCAAGGCGACGCTGACG-3') was used to generate Box-PCR profiles (Weisurg

et al. 1991). The reagents and conditions used for the Box-PCR assay are listed in Table 2 and Table 3.

Table 2 The reagents used for the Box-PCR assay.

Reagent	Volume (µL)
NZYTaq II 2x Green Master Mix	6,25
Primer	2
DNA template	1
Distilled water	Up to 25

Table 3 Conditions used for the Box-PCR assay.

Procedure		Temperature (ºC)	Time
Initial desnaturation		94 ºC	7 min
Amplification cycles (30x)	Desnaturation	94 ºC	1 min
	Anneling	53 ºC	1 min
	Extension	65 ºC	1 min
Final Extension		65 ºC	10 min
Storage		4 ºC	∞

For PCR amplification to the screening of the *mcr-1*, *mcr-3*, *mcr-4* and *mcr-5* genes the primers that are listed in Table 4 were used. The reagents required for the PCR assays are listed in Table 5.

Table 4 Characteristics of the primers used for the detection of *mcr-1*, *mcr-3*, *mcr-4* and *mcr-5* genes by PCR amplification.

Primer Name	Sequence (5' → 3')	Target Gene	Size (bp)	Reference
mcr-1-Fw *mcr-1*-Rv	CGGTCAGTCCGTTTGTTC CTTGGTCGGTCTGTAGGG	*mcr-1*	±300	(Liu et al. 2016)
mcr-3-Fw *mcr-3*-Rv	CAATCGTTAGTTACACAATGATGAAG AACACATCTAGCAGGCCCTC	*mcr-3*	676	(Jousset et al. 2018)
mcr-4-Fw *mcr-4*-Rv	ATCCTGCTGAAGCATTGATG GCGCGCAGTTTCACC	*mcr-4*	405	(Jousset et al. 2018)
mcr-5-Fw *mcr-5*-Rv	GGTTGAGCGGCTATGAAC GAATGTTGACGTCACTACGG	*mcr-5*	207	(Jousset et al. 2018)

Table 5 The reagents required for the PCR assays for the screening of *mcr-1*, *mcr-3*, *mcr-4* and *mcr-5* genes.

Reagent	Volume (µL)	Volume used for 15 samples* (µL)
NZYTaq II 2x Green Master Mix	6,25	93,75
Primer Fw	0,75	11,25
Primer Rv	0,75	11,25
DNA template	1	1
Distilled water	Up to 25	243,75

*Always prepare mix for at least 1-2 additional samples (n mix = n samples + 1 or 2).

Initially, a mixture was prepared containing 93,75 µL of NZYTaq II 2x Green Master Mix, 0,75 µL of each primer and 243,75 µL of distilled water. This mix was distributed in 15 microtubes, 24 µL in each microtube. Then, 1 µL of DNA template was added to each of the 15 microtubes. This procedure is schematically represented in Figure 26.

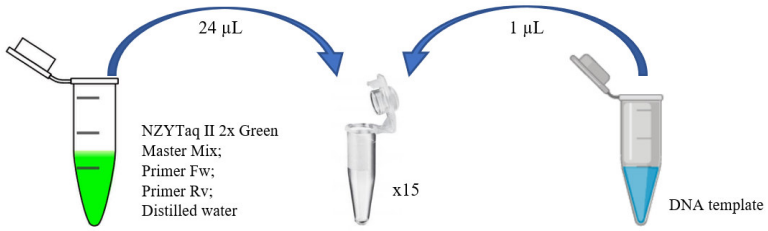


Figure 26 Schematic representation of the procedure for preparing microtubes for PCR amplification.

For the detection of the presence of the *mcr-1* gene, Ec36 strain was used as positive control. Additionally, in all assays a blank reaction was prepared without DNA template to be used as negative control. All PCRs were carried out in a Bio-Rad cycler Thermal Cycler (Bio-Rad Laboratories, Richmond, CA, USA). The conditions used for PCR amplification of the *mcr-1* gene are listed in the Table 6.

Table 6 Conditions used for the PCR assays for the screening of *mcr-1* gene.

Procedure		Temperature (ºC)	Time
Initial desnaturation		94 ºC	15 min
Amplification cycles (30x)	Desnaturation	94 ºC	30 s
	Anneling	58 ºC	90 s
	Extension	72 ºC	1 min
Final Extension		72 ºC	10 min
Storage		4 ºC	∞

The conditions used for the PCR amplification of the *mcr-3*, *mcr-4* and *mcr-5* are listed in the Table 7.

Table 7 Conditions used for the PCR amplification of the *mcr-3*, *mcr-4* and *mcr-5* genes.

Procedure		Temperature (ºC)	Time
Initial desnaturation		94 ºC	3 min
Amplification cycles (30x)	Desnaturation	94 ºC	30 s
	Anneling	56 ºC	30 s
	Extension	72 ºC	1 min
Final Extension		72 ºC	5 min
Storage		4 ºC	∞

PCR products of *mcr-1, mcr-3, mcr-4* and *mcr-5* genes screening were loaded on a 1,5% agarose gel 1X TAE (Tris-acetate-EDTA) running buffer with an electrophoresis at 90V for 60 minutes. At the end of the 60 minutes, the gel was placed in ethidium-bromide for 15 minutes and then in distilled water for 10 minutes. The results were visualized using the IMAGELab^{TM} software. In all electrophoresis carried out, 1 µL molecular weight ladder was introduced. The size of the DNA fragments was determined by comparison with the migration of fragments of known molecular weight.

A PCR assay was performed to screen the plasmid IncX4 in the *mcr-1* positive strain. The conditions used for the PCR amplification of the IncX4 plasmid are listed in the Table 8.

Table 8 Conditions used for the PCR amplification of the IncX4 plasmid.

Procedure		Temperature (ºC)	Time
Initial desnaturation		94 ºC	5 min
Amplification cycles (30x)	Desnaturation	94 ºC	30 s
	Anneling	50 ºC	30 s
	Extension	72 ºC	1 min
Final Extension		72 ºC	7 min
Storage		4 ºC	∞

4 Chapter 1. Epidemiology of colistin-resistant bacteria in CHBV

4.1 Introduction

Colistin is a cationic polypeptide antibiotic non-ribosomally synthesized by *Bacillus polymyxa* subspecies *colistinus* Koyama (Komura 1979) and was isolated, for the first time, in 1949 (Biswas et al. 2012; Bialvaei and Samadi Kafil 2015; Liu et al. 2016; Poirel et al. 2017).

Asian countries, including China, India, Japan, and Vietnam and Europe used colistin during the 1950s as a therapeutic agent in food-producing animals to improve feed efficiency and body weight gain, and as a livestock growth-promoter (Butaye and Wang 2018; Sun et al. 2018). Also, United States' Food and Drug Administration approved it in 1959, for veterinary use, to treat infections caused MDR Gram-negative bacteria, mainly Enterobacteriaceae family, in many animals. However, in the 1970s, it was replaced because it was considered both nephrotoxic and neurotoxic. At this time, health professionals started to use other antibiotics like third-generation cephalosporins and carbapenems, since they had therapeutic success and, at the same time, they were less toxic (Ezadi and Ardebili 2018; Forde et al. 2018; Sun et al. 2018).

The inappropriate and excessive use of antibiotics and the lack of new antimicrobial agents lead to the emergence of resistant strains. Currently, there are "old" antibiotics that are being studied again and re-introduced in clinical practice. Among the various revived antibiotics, colistin is the most widely used, even with a range of efficacy between 25% and 71% (Biswas et al. 2012; Shaikh et al. 2015; Ezadi and Ardebili 2018; Forde et al. 2018). Figure 27 represents the timeline for the use of polymyxins at clinical level.

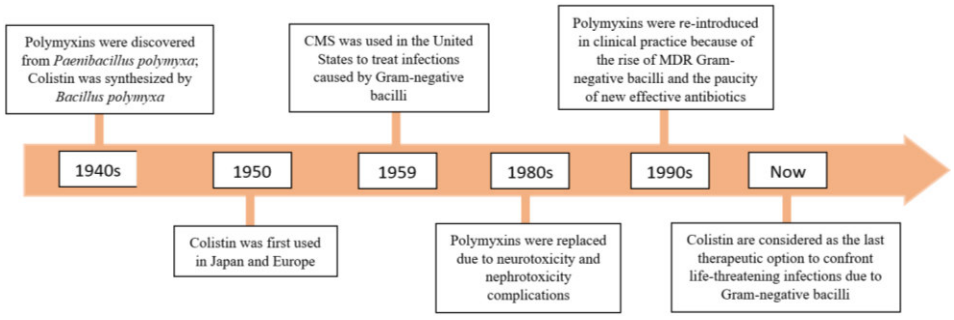


Figure 27 Timeline of the use of polymyxins at clinical level (adapted from (Ezadi and Ardebili 2018)).

Currently, colistin, more properly colistin methanesulfonate, is considered an alternative agent to treat infections caused by MDR Gram-negative pathogen since this

compound can interact with LPS of the outer membrane of these bacteria. Besides that, this antibiotic is more effective than β-lactams, quinolones, and aminoglycosides, so its' use in clinical practice is increasing globally. Certain countries, like Asia followed by Europe and America, are using this antimicrobial agent excessively, which leads to the development of high resistance rates (Bialvaei and Samadi Kafil 2015). The current dissemination of MDR Gram-negative colistin-resistant bacteria and the recent identification of plasmid-mediated mechanisms lead to the need to develop more epidemiological studies. These studies provide updated information on the prevalence of the *mcr* genes, namely the *mcr-1* gene, in both bacterial isolates of animals and humans at regional, national and global levels. It's essential to evaluate the impact of the use of polymyxins in human and veterinary medicine, to anticipate future trends in the prevalence and dissemination of plasmid-mediated colistin resistance (Poirel et al. 2017; Zurfluh et al. 2017).

4.2 Results and Discussion

4.2.1 Characterization of the collected samples and microorganisms

During the timeframe of this study (2017-2019) a total of 6189 strains were isolated. Among them, 12 non-repetitive strains of Enterobacteriaceae family (six *K. pneumoniae*, four *E. coli* and two *E. cloacae*) and one *P. aeruginosa,* which were not expected to exhibit intrinsic resistance to colistin, were collected, as shown in Figure 28. Strains with inherent resistance to colistin such as those belonging to genus *Proteus*, *Providencia* and *Serratia* were excluded from the study. The 13 strains were collected from different samples, namely, urine with and without civet, pus and blood culture, as can be seen in Figure 29.

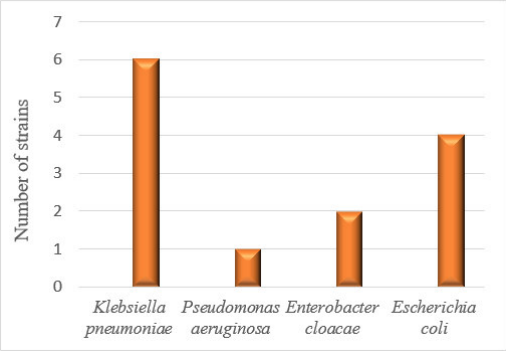


Figure 28 Number of strains non-intrinsic resistant to colistin collected.

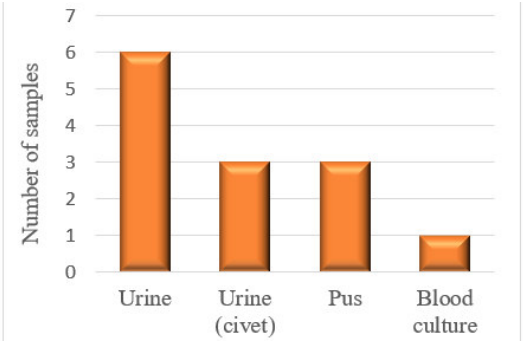


Figure 29 Number of different samples obtained during the timeframe of this study (2017-2019).

The colistin-resistant isolates collected were isolated mainly from urine, followed by pus and blood samples as show in Figure 29. This result was expected, since urine is the most common biological product to be studied in the hospital settings. Moreover, all urine samples were collected from patient with a suspected UTI. The most prevalent species isolated were *E. coli* and *K. pneumoniae* which confirms with which is described in previous reports from the same hospital (Lykholat 2018).

Demographic data of each patient with a colistin-resistant strain were collected to classify the patient as “inpatient” or “outpatient”, which is critical information to the infection control procedures to be taken. Moreover, it was important to understand the dissemination of these strains by gender, age, ward and treatment used. These data are represented in the Table 9.

Table 9 Some clinical information of the 13 patients with colistin-resistant positive samples.

Isolates	Specimen	Hospital ward	Diagnosis	Gender	Age	Admission date	Treatment used	Internment (days)
Kp574799	Urine (civet)	MED	Suspected UTI	F	71	17/04/2017		
Kp575688	Urine (civet)	SUR	Acute pancreatitis	F	61	21/04/2017		39
Kp577346	Urine (civet)	SUR		F	61	23/05/2017		
Kp593202	Blood culture	ICU	Septic screening	M	56	02/09/2017	CL MEM VAN	233
Kp174283	Urine	ER	Urosepsis	M	59	18/09/2018	-	-
Kp184772	Urine	ER	Fever	M	89	31/10/2018	CAZ IMP	*
Ec536586	Pus	ORT	Fever	M	83	16/06/2018	AMC	48
Ec567645	Urine	MED	Dehydrated	F	87	30/01/2019		
Ec176067	Urine	ER	Suspected UTI	F	73	25/09/2018	TZP	12+7
Ec186900	Urine	ER	Fever	F	86	09/11/2018		*
Pa396714	Pus	MED	Diabetic foot	F	88	27/03/2017	COZ	
Ecl556095	Pus	SUR	Operative wound	F	86	13/11/2018		*
Ecl120300	Urine	ER	Suspected UTI	M	63	03/03/2019		

MED: Medicine; SUR: Surgery; ICU: Intensive care units; ER: Emergency room; ORT: Orthopedics; F: Female; M: Male; CL: Colistin; MEM: Meropenem; VAN: Vancomycin; CAZ: Ceftazidime; IMP: Imipenem; AMC: Amoxicillin/clavulanic acid; TZP: Piperacillin/tazobactam; COZ: Cefuroxime; *The patient was still hospitalized at the time of data collection.

Considering the distribution of the samples by gender it was observed that five of the isolates were collected from samples retrieved from male patients and eight were collected from samples retrieved from female patients (Figure 30).

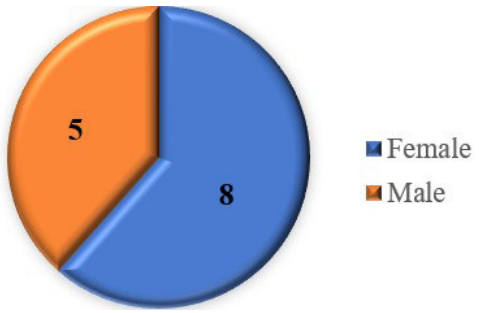


Figure 30 Sample collected distributed by gender.

These results are in accordance with the expected, since most of the samples are from the urine and, as previously reported, women are more susceptible to UTI than men. The following figure (Figure 31) shows the isolates obtained according to gender.

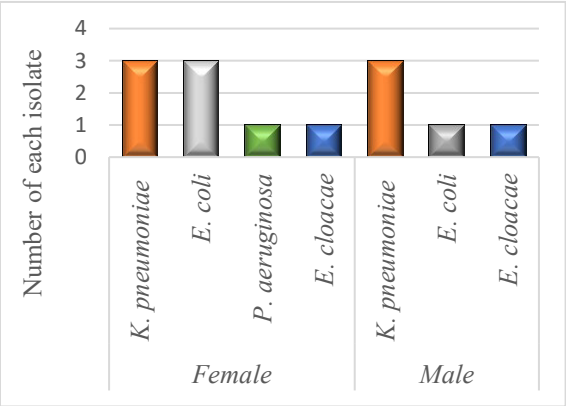


Figure 31 Number of isolates obtained according to gender.

In the female gender, the *K. pneumoniae* and *E. coli* strains occurred in the same number (3/8), while in the male gender, *E. coli* strain was only one. The most prevalent strain in the male gender was *K. pneumoniae* (3/5). These results can be explained by the fact that many of the urine samples were collected from female patients suspected to have an UTI. As previously mentioned, *K. pneumoniae* and *E. coli* are the most prevalent microorganisms in this type of infections, so it's expected that they appear in larger numbers, and higher in women than in men.

Considering Table 9, all patients studied age ranged from 56 to 89 years old. These results were somehow surprising, since it was not expected to encounter such resistant strains in relatively young patients (56 years old). At more advanced ages, the immune system becomes less effective and the patients become more susceptible to opportunistic species that don't normally cause disease. On one hand, these patients already have some antimicrobial therapy throughout its' life, so the presence of resistant strains may come from a selective pressure due to the excessive use of antibiotics. On the other hand, this also becomes more prevalent, because most of these patients are in healthcare institutions (e.g. nursing homes), and when they are discharged from the hospital they return to the institutions, where infection control measures need to be intensive, but in some cases may be deficient. Thus, they become more susceptible to recurrent infections and the development of resistance by the strains that colonize them. Another factor to consider is the transmission of genetic information between these strains, since the control of their spread becomes very difficult, as they spread beyond the hospital setting. The advanced age of most of the patients can be critical for the treatment.

4.2.2 Characterization of the sample provenance

There is a large difference between strains from a hospital setting or community-acquired. Patients were classified as inpatients or outpatients, if they were staying in the hospital or if they were only attending the ER at the moment of the sample collection, respectively, with one exception only. It was assumed that these patients represent the community.

In Figure 32, it's represented the number of isolates obtained from each hospital ward and their distribution in inpatients or outpatients.

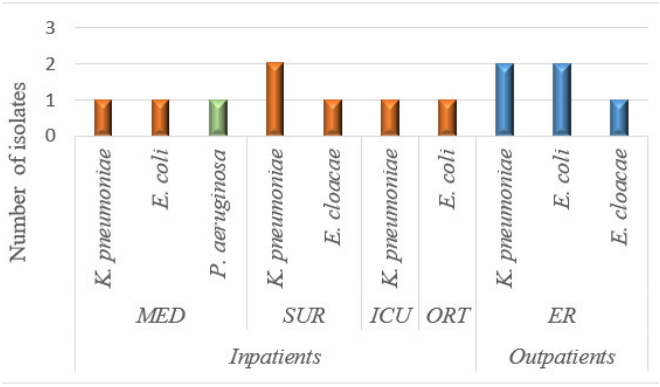


Figure 32 Number of isolates grouped by hospital ward and distribution by inpatients and outpatients.

According to the results obtained, it was observed that the same species can be obtained from patients from different wards, but also patients from the same ward may contain different species. For example, *K. pneumoniae* was present in patients of MED, ER, ICU, and ORT wards. From the MED ward beyond *K. pneumoniae* and *E. coli*, *P. aeruginosa* strains were also collected. These results show that between strains of patients from the same ward there may be exchanges of genetic material. This may be one of the explanations for resistance to colistin in different species, since resistant strains of *E. coli* can transfer resistance determinants to strains of *K. pneumoniae*, and vice-versa, for example.

Distributing the isolates obtained from the patients of each ward, by gender, it was concluded that the samples collected from patients of the MED and SUR wards are all from female patients. Patients corresponding to the ICU and ORT wards are male patients. Regarding the ER, three samples are from male and two are from female patients. All samples obtained from this ward were from urine, from patients with a diagnosis of UTI, which may reflect the higher number of samples of female patients, as previously explained.

From a total of 13 isolates, seven strains (4/6 *K. pneumoniae*, 2/4 *E. coli* and 1/2 *E. cloacae*) were recovered from inpatients in MED, SUR, ICU and ORT wards at CHBV and the other six (2/6 *K. pneumoniae*, 2/4 *E. coli* and 1/2 *E. cloacae* and 1/1 *P. aeruginosa*) from outpatient samples, attended the ER. The distribution of the isolates considering the criteria of "inpatient" or "outpatient" is represented in Table 10.

Table 10 Distribution of the isolates considering if they were collected from inpatients or outpatients.

Inpatient	Outpatient
Kp574799	Kp174283
Kp575688	Kp184772
Kp577346	Ec176067
Kp593202	Ec186900
Ec536586	Pa396714
Ec567645	Ecl120300
Ecl556095	

In addition to the information represented in Table 9, some cases were analyzed in more detail. Strains Kp575688 and Kp577346 are from the same patient, but they were collected in different dates. The patient was transferred from "Centro Hospitalar e Universitário de Coimbra" (CHUC) and was admitted to the hospital in April for 39 days in the SUR ward because of an acute pancreatitis. The strain Kp575688, which carries the *bla*$_{KPC}$ gene (data not shown), was the first to be detected. However, it was associated with colonization, since it didn't trigger any infection. Later, on May 4th, the patient returned to the hospital with suspected UTI, for which the patient was medicated and catheterized, not being hospitalized. 18 days later, the patient returns and the strain Kp577346 is recovered and associated with the UTI. Thus, the emergence of a problematic strain was notorious. The emergence of this strain causing an infection may be explained by the use of a catheter, where the strains can adhere, as well as a selective pressure caused by the antibiotic therapy used.

Strain Kp593202 was retrieved from a blood culture. In this case, the patient with this strain presented innumerable infections, for which the patient carried out immense antimicrobial therapy during his very long stay in the hospital (233 days). The emergence of a resistant strain to most available antibiotics may be associated with selective pressure from all antibiotics used.

Ec536586 colistin-resistant is an ESBL-producing *E. coli* isolated from pus of an 83-year-old male, admitted in April at the CHBV for 48 days in the ORT ward. During this

period the patient was treated with amoxicillin/clavulanic acid in monotherapy. When the patient was discharged from the hospital, he was catheterized, since the pressure ulcers that he presented did not interfere with this procedure. At the end of June, the patient returns to the hospital with a fracture of the femur neck. The bacteriological cultures related to the prothesis of femur didn't demonstrate any worrisome microorganisms. However, during the analysis of the pus sample collected from the pressure ulcer, *E. coli* strain (Ec536586), was recovered. Thus, since no intensive empiric antimicrobial therapy has been performed, antibiotic pressure was not expected to be the cause of the development of resistance by this strain.

Regarding samples from outpatients, patients carrying Kp174283 and Kp184772 strains have a very different clinical history. Although the biological product is the same (urine), strain Kp174283 comes from a patient who presented sepsis with a pyelonephritis as the starting point, reason why the patient was catheterized. The use of catheter may be at the origin of the development of this colistin-resistant strain, once it's considered one of the main risk factors for the development of bacterial infections by these microorganisms. The patient with the Kp184772 strain was hospitalized in the CHUC in 2016, with history of recurrent UTIs. This patient was constantly undergoing empiric antimicrobial therapy, mainly with imipenem and ceftazidime. With the excessive use of these antibiotics, this strain could have undergone a great selective pressure, hence the development of resistance. On the other hand, this strain carries the bla_{KPC} gene (data not shown) which may be received by HGT from CHUC colonizing bacteria, since several outbreaks of bla_{KPC} gene carrying strains have already been reported in this hospital.

As mentioned previously, in this study, there is an exception, to the criteria chosen as outpatient. The strain *P. aeruginosa* that was named as Pa396714, comes from a patient of the MED ward, however was considered from outpatient. The patient, coming from a nursing home, was attending the ER ward in January 2017, with a diabetic foot and infected pressure ulcer, where the strain Pa396714 was detected. At this point, the patient did antimicrobial therapy with amoxicillin/clavulanic acid and cefuroxime. In March 2017 the patient returns to the hospital and Pa396714 colistin-resistant was collected. Therefore, it's considered to come from an outpatient because, despite being in the MED ward in March, when he was attended in ER, in January, this strain was already present. The development of resistance by

this strain can be explained by the antibiotic pressure, since a prolonged antimicrobial therapy with an antipseudomonal agent was applied.

As noted, the number of colistin-resistant strains present in the community doesn't differ much from the number of isolates obtained from inpatients. When the strains are confined to a hospital setting, it is known that they are likely to be clones, so their characteristics should not differ much, and to some extent, it is not difficult to control their spread among the patients. However, the major concern is the strains from abroad. These strains can come from different environments and may be quite different, which hampers the development of a common treatment for these strains.

4.2.3 Results obtained from quantitative methods

4.2.3.1 MIC values for the different antibiotics tested by automated broth microdilution method Vitek2® (BioMèrieux, France).

To evaluate different characteristics of all the strains collected, several quantitative tests were carried out, namely the AST. This test was carried out through automated broth microdilution method Vitek2® (BioMèrieux, France) as explained in the material and methods section 3.5. MIC values obtained to each antibiotic tested are expressed in Table 11. The results were interpreted according EUCAST clinical breakpoints (version 0.9, 2019).

Table 11 MIC values (μg/mL) obtained to each specific antibiotic by automated broth microdilution method Vitek2® (BioMèrieux, France).

Isolate	AMP	AMC	TZP	COZ	CTX	CAZ	FEB	SxT	ERT	MEM	AMK	GEN	CIP	FOS	NIT	CL
Kp574799	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥320	≥8	≥16	≥64	≥16	≥4	≥256	NT	4
Kp575688	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥320	≥8	≥16	16	≥16	≥4	≤16	64	≥16
Kp577346	≥32	≥32	≥128	≥64	≥64	≥64	32	≥320	≥8	≥16	≥64	≥16	≥4	≤16	32	≥16
Kp593202	≥32	≥32	≥128	≥64	≥8	≥64	≥8	≥320	≥8	≥16	16	≥16	≥4	≤16	≥512	≥16
Kp174283	≥32	≥32	16	≥64	≥64	≥64	≥64	NT	0,25	≤0,25	≤2	≥16	≥4	64	128	≥16
Kp184772	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥320	≥8	≥16	8	8	≥4	64	≤16	4
Pa396714	NT	NT	8	NT	NT	2	≤1	NT	NT	0,5	4	≤1	1	NT	NT	≥16
Ecl556095	4	≥32	≤4	4	≤1	≤1	≤1	≤20	≤0,12	≤0,25	≤2	≤1	≤0,25	≥256	64	8
Ecl120300	≥32	≥32	≤4	8	NT	≤1	≤1	≤20	≤0,12	≤0,25	≤2	NT	≤0,25	64	64	≥16
Ec536586	≥32	8	≤4	≥64	≥64	16	≥64	≥320	≤0,12	≤0,25	≤2	≤1	0,5	≤16	≤16	8
Ec176067	≥32	≥32	8	≥64	≥64	16	2	≤20	≤0,12	≤0,25	≤2	≥16	≥4	≤16	≤16	≥16
Ec186900	≤2	≤2	≤4	2	≤1	≤1	≤1	≤1	≤0,12	≤0,25	≤2	≤1	≤0,25	≤16	≤16	4
Ec567645	4	4	≤4	4	≤1	≤1	≤1	≤1	≤0,12	≤0,25	≤2	≤1	≤0,25	32	32	≥16

Red color: resistant phenotype; Yellow color: intermediate phenotype; Green color: susceptible phenotype; NT: Untested. Antibiotics tested: ampicillin (AMP), amoxicillin/clavulanic acid (AMC), piperacillin/tazobactam (TZP), cefuroxime (COZ), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEB), cotrimoxazole (SxT), ertapenem (ERT), meropenem (MEM), amikacin (AMK), gentamycin (GEN), ciprofloxacin (CIP), fosfomycin (FOS), nitrofurantoin (NIT), and colistin (CL).

he antibiotics tested belong to different classes: β-lactams including penicillins (AMP, AMC and TZP), cephalosporins of second (COZ), third (CTX and CAZ) and fourth (FEB) generations, carbapenems such as ERT and MEM, aminoglycosides (AMK and GEN), quinolones (CIP), sulfonamides (SxT) and finally, the polymyxin under study – colistin.

All isolates were resistant to colistin, once this is one of the criteria of choice for this epidemiological study. The MIC values range from 4 to ≥ 16 μg/mL. These MIC values are in concordance with those obtained by Cao et al. 2018, in a study on *E. coli* strains of infected patients. They conclude that these MIC values indicate a low level of resistance to colistin. It is important to be aware of the colistin heteroresistance. This is an emerging phenomenon, in which there is a colistin-resistant subpopulation, within a susceptible population. A subpopulation can grow in the presence of ≥ 4 μg/mL of colistin, within a population with a MIC value of ≤ 2 μg/mL. This worrying phenomenon has already been reported for *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* strains (Poirel L, Jayol A 2017).

Most of the isolates studied were resistant to at least one other antimicrobial agent, and one strain (Kp574799) was resistant to 15 antimicrobials tested. All six *K. pneumoniae*, two *E. cloacae* and one *P. aeruginosa* strains analyzed were considered MDR. For *E. coli* strains, only two (Ec536586 and Ec176076) were MDR, but these demonstrated a high MIC value for penicillins, as in the work developed by Moawad et al. 2018. In this study, it was expected most isolates to be MDR, as obtained in the Li et al. 2018 study, since colistin is used when none of the other classes is effective. All *K. pneumoniae* strains were resistant to AMP, AMC, COZ, CTX, CAZ, FEB, SxT, GEN and CIP, as the results obtained by Mendes et al. 2018, where besides of 100% resistance to third and fourth generation cephalosporins and monobactams, *K. pneumoniae* isolates were resistant to CIP (96%), GEN (88%), FOS (83%) and SxT (79%). For these strains, intravenous FOS is currently used, since it's indicated for UTIs (Qamar et al. 2017), however, three of them already exhibit resistance to this antimicrobial, which makes it even more difficult to choose antimicrobial therapy for these pathogens.

A similar resistance profile is shown among the six *K. pneumoniae* strains collected. Regarding the two *E. cloacae* isolates, their resistance profile is similar, since MIC values are the same for almost all the antibiotics tested. Among the four *E. coli* strains, the resistance profile of Ec536586 and Ec176067 is similar: they are resistant to almost the same antibiotics. However, Ec176067 comes from an outpatient while Ec536586 from an inpatient of the ORT ward. At the temporal level they only three months apart. The other two *E. coli* strains (Ec186900 and Ec567645) are susceptible to all antibiotics, except for colistin and the MIC values differ only for AMP, AMC, COZ, FOS and NIT. All the *P. aeruginosa*, *E. cloacae* and *E. coli* isolates were sensitive to the carbapenems, which is a similar result obtained by Moawad et al. 2018. The strain *P. aeruginosa* is difficult to compare with the other isolates since it doesn't belong to the Enterobacteriaceae family and the antibiotics tested by Vitek2® (BioMèrieux, France) aren't the same. In Figure 33 is graphically represented the number of isolates resistant to each one of the antibiotics tested.

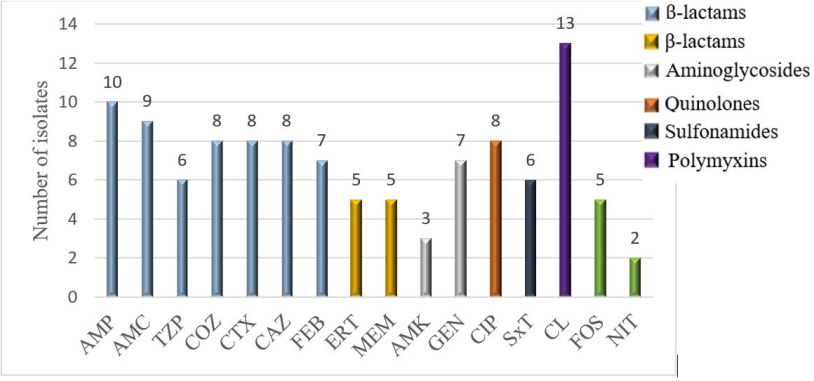


Figure 33 Number of isolates resistant to each one of the antibiotics tested.

The classes of antibiotics to which the microorganisms showed higher levels of resistance were the β-lactams, especially AMP (10/13) as in the study by Lu et al. 2018 and AMC (9/13). Followed by quinolones (8/13) and one of the aminoglycosides – GEN (7/13). Almost all isolates were resistant to penicillins, which indicate that the use of this antibiotics are compromised. The high number of microorganisms resistant to a certain antibiotic may be related to the long therapeutic availability of this antibiotic. The antibiotics for which less resistance was shown were AMK and NIT. Of the 13 isolates, five showed resistance to FOS, which may indicate that this antibiotic may be compromised shortly, in clinical practice.

Analyzing in detail the resistance to carbapenems (ERT and MEM), the results showed that the five isolates exhibiting a resistant profile belonged to *K. pneumoniae* species.

It was later confirmed, in another study with the same strains, that the *bla*$_{KPC}$ gene was present. This may justify resistance to carbapenems, since in the susceptible strain (Kp174283) the gene was not detected. Looking for plasmid-encoded resistance in this group of isolates might be crucial for management of patients and epidemiological purpose, since colistin is one of the last therapeutic options to treat carbapenem-resistance Enterobacteriaceae (Jousset et al. 2018).

Among the isolates resistant to penicillins (AMP, AMC and TZP) and cephalosporins (COZ, CTX, CAZ and FEB) three are ESBL-producers – two *E. coli* and one *K. pneumoniae*. As noted in the general introduction, these enzymes are most commonly produced by members of the Enterobacteriaceae family, namely, *K. pneumoniae* and *E. coli*, as demonstrated in these results. However, these enzymes do not have the capacity to hydrolyze carbapenems as shown in Table 11, ESBL-producing strains (Kp174283, Ec536586 and Ec176067) are susceptible to carbapenems. Among the 13 strains, seven were considered non-ESBL-producers and for the other three (Kp575688, Pa396714 and Ecl120300) it was not possible to obtain this information. However, it is important to remind that a negative result for ESBL, doesn't exclude the presence of an ESBL hidden by a β-lactamase AmpC. In addition to β-lactams resistance, these strains may also have other enzymes that confer resistance to other classes of antibiotics, such as aminoglycosides (AMK and GEN), which is also demonstrated in the results presented in Table 11.

4.2.3.2 MIC values obtained by MICRONAUT-S broth microdilution Colistin MIC test (Merlin Diagnostika)

Compared to dilution methods, automated broth microdilution method Vitek2® (BioMèrieux, France) presents a low sensitivity to detect colistin-resistant Gram-negative isolates and isn't reliable to detect heteroresistant subpopulations. Therefore, the result of automated method Vitek2® (BioMèrieux, France) for the colistin-resistant strains must be confirmed by another method. For this confirmation, the concentration gradient test - MICRONAUT-S broth microdilution Colistin MIC test was used, in accordance with the EUCAST recommendations (Version 9.0, 2019). These results were acquired through the routine practices of the CHBV Microbiology Laboratory. MIC values for four of the 13 isolates are shown in Table 12.

Table 12 MIC value (µg/mL) of four strains obtained by MICRONAUT-S broth microdilution Colistin MIC test (Merlin Diagnostika).

Isolate	MIC value (µg/mL)
Kp174283	16
Kp184772	4
Ec176067	16
Ec186900	8

The MIC values obtained by MICRONAUT-S broth microdilution Colistin MIC test (Merlin Diagnostika) are coincident with those obtained by automated broth microdilution method Vitek2® (BioMèrieux, France), except for the MIC value of strain Ec186900. In this case, the MIC value obtained by this method is higher than that obtained by automated method Vitek2® (BioMèrieux, France). This result reinforces the necessity to confirm the results obtained with the automated method Vitek2® (BioMèrieux, France) for all colistin-resistant strains.

4.2.4 Results obtained from qualitative method NG-Test MCR-1 (NG Biotech, France)

The qualitative method NG-Test MCR-1 (NG Biotech, France) was used to detect the presence of the MCR-1 enzyme among the 13 isolates collected. In Figure 34 is represented an example of the results obtained.

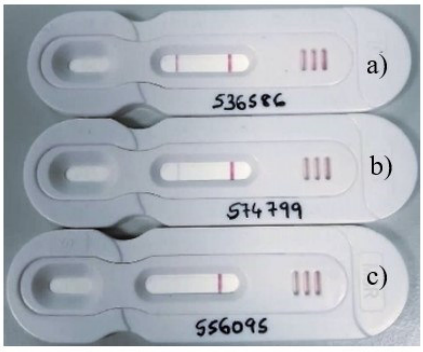


Figure 34 Example of results obtained by NG-Test MCR-1 (NG Biotech, France). a) positive result; b) positive result; c) negative result.

The results were negative for all *E. cloacae* strains (e.g. Ecl556095 shown in the Figure 34) for *P. aeruginosa,* five *K. pneumoniae* and three *E. coli* strains. A positive result was

obtained for one *K. pneumoniae* strain (Kp574799) and for one *E. coli* strain (Ec536586), as shown in Figure 34. These results lead one to hypothesize that the resistant phenotypes exhibited by the majority of the strains, of this study, may originate from intrinsic mechanisms, such as mutations, or changes in chromosomal genes, since the presence of MCR-1 enzyme produced by *mcr-1* plasmid-mediated, was not detected.

4.2.5 Results obtained from genotypic methods – PCR amplification

4.2.5.1 Bacterial typing – Box-PCR amplification

In this study, Box-PCR was used for bacterial typing of 10 strains (six *K. pneumoniae*, four *E. coli*), since this technique allows the typing of many isolates in a simple and fast way. Some of the results obtained by the typing with Box-PCR can be seen in Figure 35.

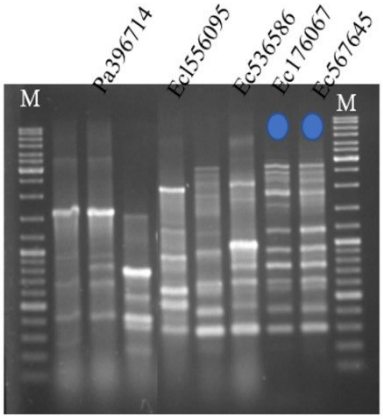


Figure 35 Box-PCR profile of some isolates.

The analysis of the results obtained, to the *E. coli* strains, with the Box-PCR showed the presence of two clones and two other different patterns.

Normally, when the isolates share the same DNA profile or fingerprint, they are epidemiologically related and were probably were originated from the same clone and transmitted between patients by a common source. The Box-PCR technique allowed to group the different isolates in identical profiles, however, it doesn't guarantee that they are clones. This technique doesn't detect MGE as transposons, genomic islands, plasmids, among others, hence, when resistance is associated with them, it may not be detected. Thus, it's explained that isolates with an identical profile, doesn't necessarily have an identical antibiogram, as it is verified in this study (see Table 11). To ensure that two or more strains are effectively clones, more bacterial typing techniques such as REP-PCR and ERIC-PCR are needed.

Regarding the *K. pneumoniae* strains, there were no clones detected, there were six different profiles. In this case, these isolates are epidemiologically unrelated once they have distinctly different patterns. Nonetheless, all these strains exhibited a similar antibiogram.

4.2.5.2 Screening of *mcr-1*, *mcr-3*, *mcr-4* and *mcr-5* genes by PCR amplification

The 13 isolates phenotypically confirmed as colistin-resistant by automated broth microdilution method Vitek2® (BioMèrieux, France) were tested by PCR for the detection of the *mcr-1*, *mcr-3*, *mcr-4* and *mcr-5* genes as explained in the material and methods section 3.8.1. The *mcr-1* gene is globally distributed, however since *mcr-3*, *mcr-4*, and *mcr-5* variants have been recently described, it was considered appropriate to also screen these variants among the strains included in this study (García et al. 2018; Wang et al. 2018).

The *mcr-1* gene was detected in two strains - **Ec536586** and **Kp574799** - represented as number six and 13 in the Figure 36, respectively. These results agree with the results obtained by the qualitative method NG-Test MCR-1 (NG Biotech, France).

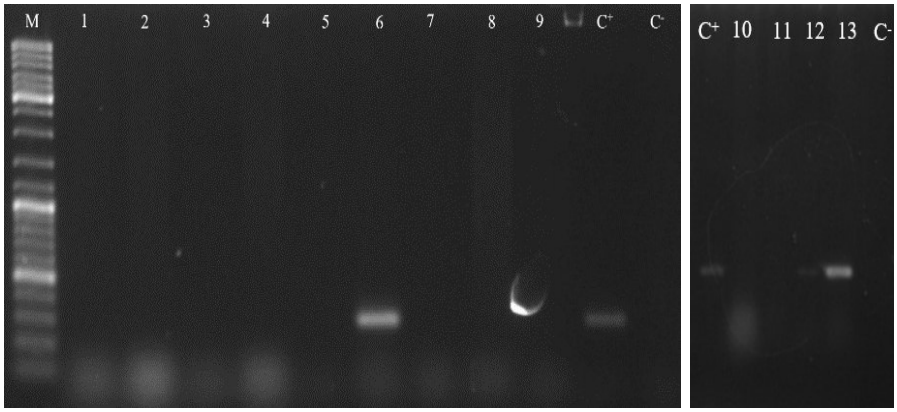


Figure 36 Results obtained for the screening of *mcr-1* gene. C^+: Positive control – Ec36 (Tacão et al. 2017); C^-: Negative control; 1- Kp577346; 2- Kp593202; 3- Kp575688; 4- Kp174283; 5- Ec176067; 6- Ec536586; 7- Pa396714; 8- Kp184772; 9- Ecl556095; 10- Ec186900; 11- Ec567645; 12- Ecl120300; 13- Kp574799;

Ec536586 strain was obtained from a patient who was hospitalized in the ORT ward, as explained in section 4.2.2. The *E. coli* (Ec36) strain, analyzed by Tacão et al. 2017 was also collected from a patient of this hospital ward. Regarding this information, one can hypothesize that Ec536586 strain may be present in the CHBV ORT ward, since the Ec36 strain was previously identified in this hospital ward with the same gene.

Kp574799 strain, as the strains studied by Mendes et al. 2018, is a Carbapenemase-producing *K. pneumoniae.* This strain and Ec36 were collected from the same patient months apart and both carry the *mcr-1* gene. In the case of Ec36 strain its genetic context was studied by Tacão et al. 2017. The presence of bla_{KPC} gene in strain Kp574799 was also confirmed (data not shown) which might suggest a similar genetic context.

The major risk factors for infection associated with the emergence of strains positive for *mcr-1* gene indicated by Wang et al. 2017 are: be of the male gender, immunosuppressed, or having received antibiotics in the past three months. Use of carbapenems and fluoroquinolones in the previous three months was particularly associated with increased risk. Patient age was not associated with *mcr-1*-positivity (Wang et al. 2017). In the present study, both patients carrying strains positive *mcr-1* were immunocompromised and received antibiotics in the previous three months. However, for the patient carrying strain Ec536586 antimicrobial therapy was not relevant. On the other hand, they have different genders.

Contrary to the results obtained by Zhang et al. 2018, where it was detected a high prevalence of *mcr-4* gene in human samples, in this study, the *mcr-3*, *mcr-4* and *mcr-5* genes were not detected in any of the strains.

According to the literature, the *mcr-1* gene has already been detected in members of Enterobacteriaceae family from healthy individuals and/or patients in over 40 countries across five continents, both in endemic outbreaks and in sporadic cases (Yin et al. 2017; Cao et al. 2018; Sun et al. 2018). SENTRY program report that among the colistin-resistant isolates collected between 2014 and 2015 from different countries, 4,9% isolates were *mcr-1*-positive, which represented a high prevalence (>30.0% of colistin-resistant isolates). All these isolates were *E. coli* strains, distributed in 10 countries around the globe. These were mainly associated with bloodstream infections, skin and skin structure infections, UTIs, IAI and respiratory tract infections. The Ec536586 strain is very similar to the isolate collected by SENTRY program from USA. They are susceptible to TZP, MEM, GEN, AMK, NIT and FOS, and, at the same time, they are resistant to SxT and CAZ. In addition, both are ESBLs producers.

In the multicenter longitudinal study, developed by Quan et al. 2017, with 1495 *E. coli* and 571 *K. pneumoniae* isolates, collected from patients with bloodstream infections, only 1% (20/1495) of *E. coli* strains and ~0,2% (1/571) of *K. pneumoniae* strains were positive

for *mcr-1* gene. Wang et al. 2018 also reported the presence of *mcr-1* and *mcr-3* genes in *K. pneumoniae* isolates at relatively low detection rates. Besides that, in the SENTRY program, all *K. pneumoniae* were *mcr-1*-negative. These results could indicate that the predominant host for *mcr-1* gene is *E. coli* as observed in the results of this study.

Unlike the results obtained by Guerin et al. 2016, in which two *E. coli* isolates *mcr-1*-positive showed a high resistance to carbapenems due to the production of an CTX-M-3 ESBL, the *E. coli* isolate *mcr-1*-positive, in this study, is sensitive to the carbapenems. Also, the resistance profile of this strain is different to that obtained for strain Ec36, studied by Tacão et al. 2017, since Ec36 had resistance genes to β-lactams, carbapenems and aminoglycosides, whereas Ec536586 is sensitive for the last two. However, the results obtained are in agreement with those obtained by Cao et al. 2018, since the *mcr-1*-positive strains studied by them were also sensitive to carbapenems. In addition, these authors also found ESBL genes conferring resistance to cephalosporins and penicillins in *mcr-1*-positive strains, which was also observed in this study, since the *E. coli* strain bearing the *mcr-1* gene is also resistant to these classes of drugs. The presence of the other genes might indicate a selective role for *mcr-1* gene and lead to its dissemination (Cao et al. 2018; Rayanne et al. 2018). Poirel et al. 2017 concluded that the presence of the *mcr-1* gene in *E. coli* strains increases 4- to 8-fold the MIC value of colistin, however, in this work, this was not observed. On the contrary, the *mcr-1*-positve strains had a lower MIC value (4 and 8 μg/mL) than most *mcr-1*- negative (≥16 μg/mL).

Guerin et al. 2016 in a study with *E. cloacae*, *K. pneumoniae*, *K. oxytoca* and *C. freundii* isolates conclude that the *E. cloacae* isolates belonged to a subspecies that was classified as naturally resistant to colistin, and for *K. pneumoniae*, *K. oxytoca* and *C. freundii* isolates no putative phosphoethanolamine encoding gene was found, suggesting that the colistin resistance acquired was chromosome encoded. These results may explain the development of resistance by the two strains *E. cloacae* and five *K. pneumoniae* in this study.

Another mechanism that may be behind the development of resistance acquired to colistin by carbapenem-resistant strains *K. pneumoniae* is the changes in the several genes participating in bacterial cell membrane remodeling, mainly *mgrB* gene. The inactivation of this gene triggers negative feedback regulator of the PhoPQ two-component system, which active Pmr system. This system is responsible for modification of the LPS, preventing the

action of colistin. Currently, different genetic alterations have been observed, such as insertional inactivation by various insertion sequences (e.g. IS5-like type), point mutations and small or even large deletions of the *mgrB* locus (Cannatelli et al. 2014; Bardet and Rolain 2018). Alterations of *mgrB* were associated with a relatively broad range of colistin MIC values, from 4 to 64 μg/mL (Cannatelli et al. 2014), which may explain results obtained in this work, since some strains have MIC value of 4 μg/mL but other have a MIC value of ≥ 16 μg/mL. In general, acquired resistance to colistin has been associated with LPS modifications by several mechanisms such as the addition of cationic groups, loss of the LPS and, the overproduction of capsule polysaccharide. Mutations in the outer membrane porins and overexpression of efflux pump systems may also be involved.

This study reveals that the prevalence of colistin-resistant strains of human samples from clinical infections in the hospital settings remains low. It's important to evaluate the dissemination of bacteria carrying the *mcr-1* gene in CHBV, namely in ward environment, medical equipment since both *mcr-1*-positive strains were from inpatients.

4.2.5.3 Screening of IncX4 plasmid

According Tacão et al. 2017, that reports a study with a strain from the same hospital, the *mcr-1* gene can be found in an IncX4 plasmid. However, in the present work that was not verified. Nonetheless, since the initial detection of *mcr-1* gene in the IncI2-type (pHNSHP45) plasmid by Liu et al. 2016, the diversity of *mcr-1*-harboring plasmids is increasing, with more than 10 types of plasmids. According to GenBank, the predominant replicon types of plasmid carrying *mcr-1* are IncI2, IncX4, and IncHI2, whilst some belong to the groups IncF, IncN, IncP, IncQ and IncX. The identification of the *mcr-1* gene on several plasmid backbones from different strains and from different geographic locations, suggests that its spread corresponds to multiple genetic events that can occur independently in distantly related geographical areas.

Thus, most of plasmids circulated in multiple species of Enterobacteriaceae in the world (Nordmann and Poirel 2016; Sun et al. 2018). Since the *mcr-1* gene may be present in any of the plasmids, other than IncX4, it may be possible that it can be what is happing in this strain. Further studies will be needed to ascertain in which plasmid the *mcr-1* gene of strain Ec536586 is present.

4.3 Conclusion

Although colistin has been and still is widely used in veterinary medicine and is used at increasing frequencies in human medicine, the rates of resistance development in human isolates remains low. The resistance exhibited by the strains included, apart from two isolates, in this study was not due to the presence of *mcr*-1 gene. One can hypothesize that it may be due to chromosomal mutations. However, the detection of plasmid-mediated colistin-resistant genes that can be transmitted between the animals, natural environment, human body, and different strains through different MGEs, represents a great disadvantage in the revival of colistin. Therefore, this calls into question the use of this antibiotic as the last line of defense against carbapenem-resistant bacteria. Acquisition of *mcr-1* gene by Carbapenemases-producing Enterobacteriaceae from hospital setting, may lead to the development of PDR bacteria, which compromises the treatment of infectious disease (Yin et al. 2017; Cao et al. 2018).

Thus, these results suggest it is necessary to implement effective measures to monitor the spread of these genes and highlight the importance of active surveillance efforts as well as an adapted protocol in clinical Microbiology laboratories to effectively detect colistin- and carbapenem-resistant pathogens. Besides that, it's important to reconsider the use of in-feed colistin in veterinary medicine at a worldwide level, to try to preserve the effectiveness of this drug for society, now and into the future.

5 Chapter 2. *In vitro*, to evaluate, the efficacy of Ceftolozane/tazobactam strips against *Pseudomonas aeruginosa* and β-lactamases producing Enterobacteriaceae, which cause UTI

5.1 Introduction

Gram-negative bacteria, mostly lactose fermenters *E. coli* and *K. pneumoniae* are the most prevalent in UTIs since they are major colonizers of the intestinal reservoir and ascend from the fecal flora to the urinary channels. Colonization with MDR Gram-negative bacteria such as ESBL-producing and Carbapenem-resistant Enterobacteriaceae is often associated with community-acquired infections, commonly among residents in long-term care facilities. These institutions are considered enormous reservoirs of these pathogens and could be the origin of their transmission to the hospitals, hindering the therapeutic options (Kaye and Pogue 2015; Khoshnood et al. 2017; Saran et al. 2019).

Carbapenems have been the most frequently recommended antibiotics for the treatment of infections caused by MDR Gram-negative pathogens because of their resistance to ESBLs hydrolysis. These antibiotics are even considered "last-line" for the treatment of UTIs. However, the worldwide consumption of carbapenems has increased significantly over the past two decades, particularly in developing countries. Carbapenem resistance in key Gram-negative pathogens is now a rapidly developing phenomenon, facilitated by globalization (Saran et al. 2019).

New combinations of β-lactam/β-lactamase inhibitor, that include classic β-lactamases inhibitors (e.g., sulbactam, clavulanate, tazobactam), could be one solution to overcome the problem of resistance (Kaye and Pogue 2015; Montravers and Bassetti 2018). Cef/taz was approved in 2014 by the United States Food and Drug Administration as ZERBAXA® as one β-lactam/β-lactamase inhibitor combination. It's indicated as a powerful antimicrobial therapy alternative to carbapenems for the treatment of lower cUTI or pyelonephritis caused by hard treatment uropathogens like MDR Gram-negative bacteria. In combination with metronidazole may also be used for the treatment of complicated IAI by anaerobic microorganisms (Cluck et al. 2015; van Duin and Bonomo 2016; Theuretzbacher 2017; Giacobbe et al. 2018; Sheu et al. 2018).

The introduction of these new combinations into hospital formularies should be careful to avoid the overuse in patients with infections caused by more susceptible organisms (van Duin and Bonomo 2016).

5.2 Results and Discussion

5.2.1 Characterization of the collected samples

A total of 67 non-repetitive clinical Gram-negative isolates were collected during the timeframe of this study, September to November 2018, from different wards of the CHBV. These isolates were selected according to selection criteria previously reported. Since the infection under study was a UTI, the biologic product selected was urine and only one sample collection was by the catheter.

During the timeframe of this study, samples from positive urine cultures, as explained in the material and methods section 3.3, were distinguished in nosocomial and non-nosocomial. A nosocomial sample is obtained from an infection acquired in a hospital setting. The sample was considered nosocomial when there was a 72h difference between the patient's admission date and the sample culture date. The remaining samples were considered non-nosocomial. It was assumed that these samples represent the community. The data collected are shown in Figure 37.

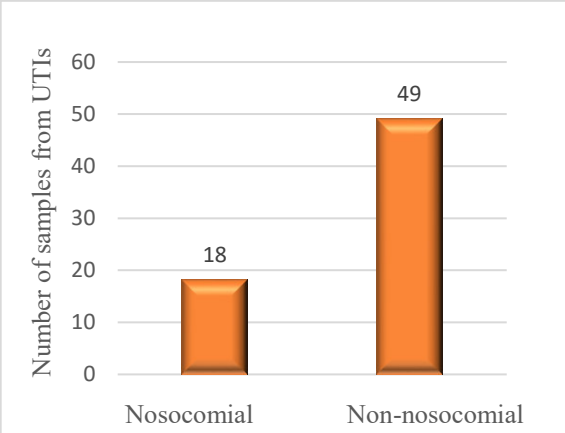


Figure 37 Distribution of positive urine cultures between strains from the hospital settings - nosocomial - and from out of hospital - non-nosocomial.

Currently, it's very important to monitor non-nosocomial infections, because these are increasing more and more, as shown in Figure 37 by the high number of samples collected as non-nosocomial. If most patients received in CHBV present non-nosocomial UTIs caused by pathogens resistant to available antibiotics, it means that the spread of these strains is far beyond the hospital setting. Regarding treatment, it becomes more difficult to apply adequate

therapy because there is a huge diversity of microorganisms with different mechanisms of resistance.

5.2.2 Characterization of the agents causing UTI

The urine samples studied revealed a diversity of microorganisms within the Enterobacteriaceae family, as shown in Figure 38. In a total of the 67 isolates, there were six *P. aeruginosa* and 61 Enterobacteriaceae (28 *E. coli*, 23 *K. pneumoniae*, two *K. oxytoca*, two *E. aerogenes*, three *E. cloacae*, one *S. marcescens*, one *C. freundii*, one *M. morganii*). Many of the samples identified in the Microbiology Laboratory contained *Proteus* species, mainly *P. mirabilis*, however, these microorganisms weren't considered due to their ability to produce swarming effect. Since the activity of Cef/taz was performed on Mueller Hinton Agar plate as explained in the material and methods section 3.7.1, it has no ability to inhibit this effect, so results wouldn't be possible to observe.

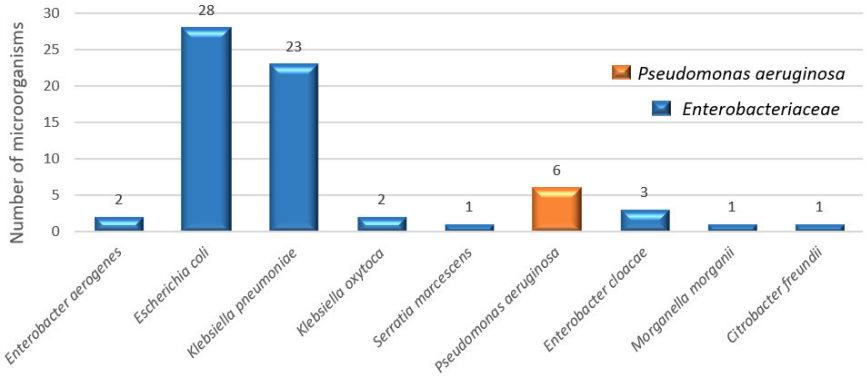


Figure 38 Number of each microorganism isolated from the samples collected, causing UTIs.

Of all isolated microorganisms belonging to the Enterobacteriaceae family, the most prevalent species was *E. coli,* followed by *K. pneumoniae* which is in accordance with the literature (Kaye and Pogue 2015; Theuretzbacher 2017) and with the recent results obtained by (Lykholat 2018) and (Roxo 2015) in the same hospital. These species were followed by strains of *P. aeruginosa*.

One of the most important mechanisms of resistance by Enterobacteriaceae family is the production of ESBLs enzymes, as reported in the general introduction. In the literature, the rate of infections caused by these bacterial strains varies between 25% to >90%, depending on the hospital ward and geographical location (Saran et al. 2019). ESBL-producers were selected by an indication of the automated broth microdilution method

Vitek2® (BioMèrieux, France), as explained in the material and methods section 3.5. The results are shown in the Figure 39.

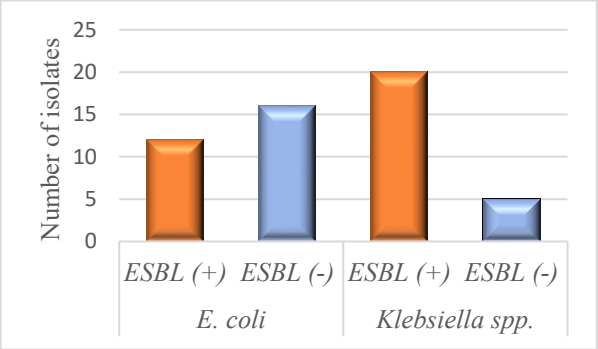


Figure 39 Number of microorganisms ESBLs-producers, among *E. coli* and *Klebsiella* spp. isolates.

Among Enterobacteriaceae members, ESBL production was detected in 32 strains. Among them, 12 strains were *E. coli* and 20 strains were *Klebsiella* spp. (19 *K. pneumoniae* and one *K. oxytoca*). The number of *Klebsiella* spp. was higher than *E. coli* ESBL-producers, as verified in the study by Lykholat 2018 in the same hospital. It was expected that ESBL-producers were mostly *E. coli* and *K. pneumoniae* strains, because within the Enterobacteriaceae family, these species are the ESBL-producers in higher numbers.

To *P. aeruginosa,* multidrug efflux pumps and loss of outer membrane porin OprD are the most common resistance mechanisms (Haidar et al. 2017), therefore, the production of ESBL isn't evaluated for these microorganisms. None of these strains was a carbapenemase-producer.

Regarding the number of classes of antibiotics to which different microorganisms are resistant, among the isolates selected, 36 were MDR isolates, 29 were Non-MDR and two were XDR. Results are shown in Figure 40. Table 13 show the different MDR, Non-MDR and XDR microorganisms. EUCAST breakpoints (version 9.0, 2019) were considered in all cases, and consensus recommendations were used to define MDR and XDR phenotypes.

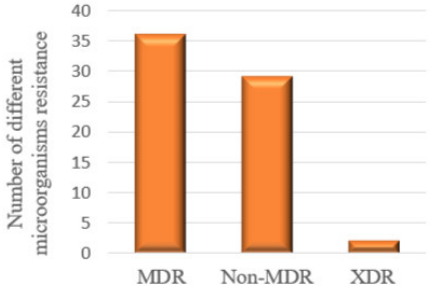


Figure 40 Number of different microorganism's resistance.

Table 13 Different MDR, Non-MDR and XDR microorganisms.

	MDR	Non-MDR	XDR
E. coli	15	13	0
K. pneumoniae	18	5	0
K. oxytoca	0	2	0
S. marcescens	1	0	0
E. cloacae	0	3	0
M. morganii	1	0	0
C. freundii	0	1	0
E. aerogenes	1	1	0
P. aeruginosa	0	4	2

Regarding the data collected it is possible to conclude that most of the microorganisms isolated are MDR Gram-negative bacteria. Figures 41 and 42 schematically represent the distribution of isolates from samples considered nosocomial or non-nosocomial, according to MDR, Non-MDR and XDR phenotypes.

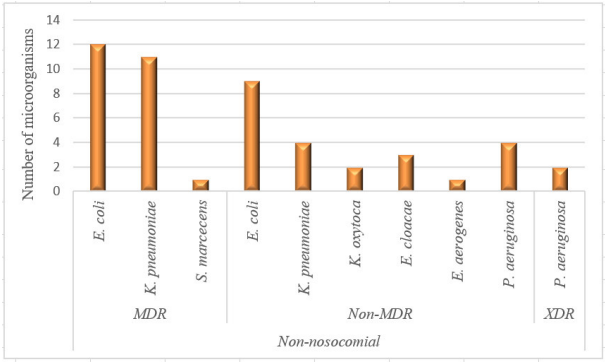


Figure 41 Numbers of isolates distributed by MDR, Non-MDR and XDR phenotype, among the samples considered non-nosocomial.

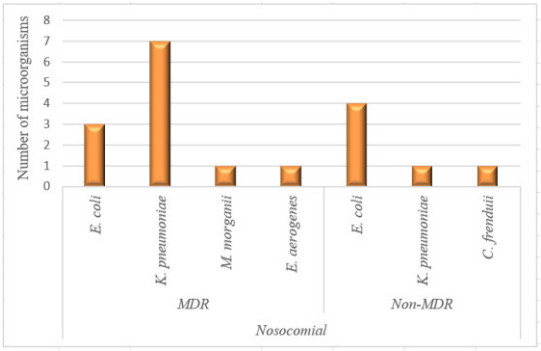


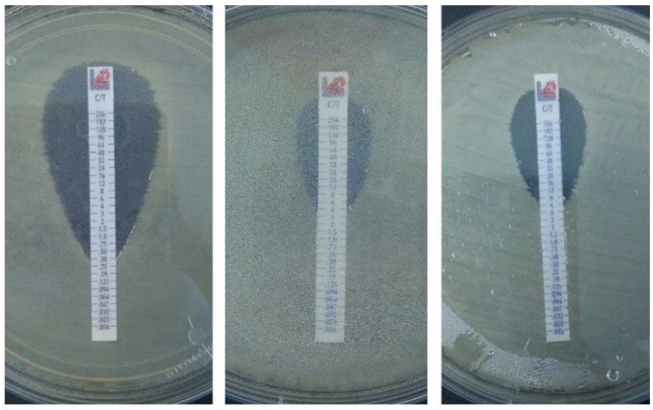
Figure 42 Number of isolates distributed by MDR and Non-MDR phenotype, among the samples considered nosocomial.

Among the 49 isolates collected from a positive urine culture and considered as non-nosocomial, according to the selected criteria, 24 (24/36) are MDR microorganisms, being mainly *E. coli* and *K. pneumoniae* (12 (12/28) *E. coli*, 11 (11/23) *K. pneumoniae* and one *S. marcescens*). 23 of the remaining positive urine cultures are associated with Non-MDR strains, being also in higher number strains of *E. coli* (9/28) followed by *K. pneumoniae* (4/23) and *P. aeruginosa* (4/6), and two with XDR microorganisms, also *P. aeruginosa* strains. With the results obtained it is concluded that the *E. coli* and *K. pneumoniae* are the most prevalent strains in samples considered non-nosocomial. Thus, it is possible to consider that these are the two most widely disseminated strains in the community, as previously reported.

Among the 18 samples of urine cultures considered nosocomial, 12 (12/36) are MDR bacteria, but in this case, the most prevalent strain was *K. pneumoniae* (three (3/28) *E. coli,* seven (7/23) *K. pneumoniae*, one *M. morganii* and one (1/2) *E. aerogenes)*. The isolates collected from the other six samples are Non-MDR, among them, there are mainly *E. coli* strains (four (4/28) *E. coli*, one (1/23) *K. pneumoniae* and one *C. freundii*) and in these samples, isolates with XDR phenotype were not obtained. Considering the results obtained, it is observed that the main MDR bacteria disseminated in the patients from various wards of CHBV is *K. pneumoniae*, followed by *E. coli*. The presented result confers with the current problem of public health - emergence of MDR Gram-negative bacteria in hospital infections, namely UTIs. These microorganisms are very worrisome, since they make it difficult for antimicrobial therapy, and even, in some cases, even without any option.

5.2.3 Activity of Cef/taz test strip

In the analyzed period, all of 67 non-repetitive isolates, which were in the spectrum of activity of Cef/taz were test to Liofilchem Ceftolozane-tazobactam MIC Test Strip (Liofilchem, Italy) as explained in the material and methods section 3.7.1. The results obtained, and their interpretation are shown in Figure 43 and Figure 44. The MIC value obtained for the different isolates is represented in Table 14. The MIC values ranges for the 67 organisms were 0,25/4 to ≥ 256/4 µg/ml.



Escherichia coli
0,38 µg/mL
Sensitive

Klebsiella pneumoniae
4 µg/mL
Intermediate

Enterobacter aerogenes
8 µg/mL
Resistant

Figure 43 Results obtained by using Cef/taz strips and their interpretation.

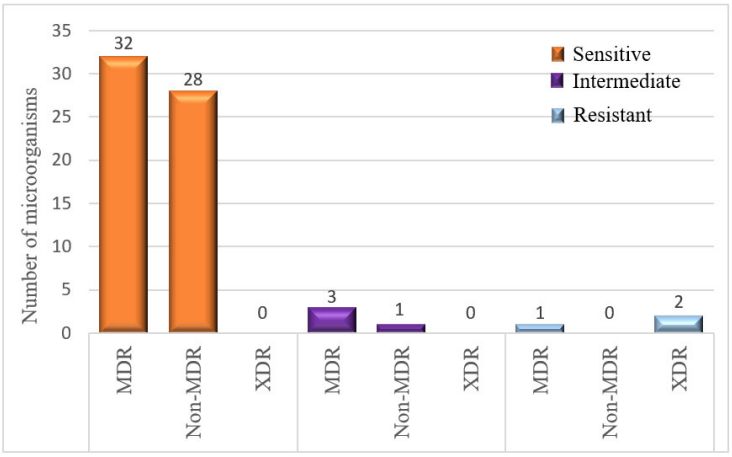


Figure 44 Number of microorganisms sensitive, intermediate and resistant to activity of Cef/taz.

Table 14 MIC value (µg/mL) obtained for Cef/taz test against Enterobacteriaceae members and *P. aeruginosa*.

Isolates	MIC frequency (µg/mL)												
	0,25	**0,38**	**0,5**	**0,75**	**1**	**1,5**	**2**	**3**	**4**	**6**	**8**	**≥16**	**≥256**
E. coli	1	10	6	2	5	1	2	-	-	1	-	-	-
K. pneumoniae	-	-	2	5	7	3	1	4	2	-	-	-	-
K. oxytoca	1	-	-	-	-	-	-	-	-	1	-	-	-
S. marcescens	-	-	-	-	1	-	-	-	-	-	-	-	-
E. cloacae	-	1	1	1	-	-	-	-	-	-	-	-	-
M. morganii	-	-	1	-	-	-	-	-	-	-	-	-	-
C. freundii	-	-	-	1	-	-	-	-	-	-	-	-	-
E. aerogenes	-	-	1	-	-	-	-	-	-	-	1	-	-
P. aeruginosa	-	-	1	1	-	1	1	-	-	-	-	1	1

Among the 67 isolates collected, 60 exhibited a sensitive result for Cef/taz strip. Among these, 32 were previously classified as MDR (14 *E. coli*, 16 *K. pneumoniae*, one *S. marcescens,* and one *M. morganii*) and 28 Non-MDR (five *K. pneumoniae* isolates, one *K.*

oxytoca, one *C. freundii*, three *E. cloacae*, one *E. aerogenes*, 13 *E. coli,* and four *P. aeruginosa)*. Four exhibited an intermediate result - three MDR isolates (one *E. coli* and two *K. pneumoniae)* and one *K. oxytoca* Non-MDR. Finally, three isolates exhibited a resistant phenotype - XDR *P. aeruginosa* and one MDR *E. aerogenes*.

The lower MIC value obtained was 0,25 μg/mL by one *E. coli* and one *K. oxytoca* strain. The *E. coli* strain is Non-MDR and doesn't produce ESBLs, while *K. oxytoca* is Non-MDR but is ESBL-producer. Among ESBL-producing *E. coli* strains (see Figure 39), susceptibility was detected in 11 of the 12 isolates. For *Klebsiella* spp. isolates was detected in 17 of the 20 isolates. The activity of Cef/taz against ESBL-producing strains is high, as demonstrated in the results obtained by Pazzini et al. 2019. In this case, out of the 147 ESBL-producing isolates, 77.6% were susceptible for Cef/taz and these isolates showed a higher susceptibility rate (85%), according to the EUCAST clinical breakpoints (version 9.0, 2019) for Enterobacteriaceae. These results can be justified by the fact that the ESBL enzymes are readily inactivated or inhibited by tazobactam. Synergistic effects observed with Cef/taz against ESBL-producing isolates could be due, at least in part, to the inhibitory activity of tazobactam, as expected.

Similar results were reported by Shortridge et al. 2017, they evaluated the activity of Cef/taz in 966 *E. coli* and 369 *K. pneumoniae* isolates, and 92.2% and 5.1% of strains were susceptible, respectively. In other study performed by Dandan et al. 2019, Cef/taz showed high activity against most Enterobacteriaceae members (inhibited 74.7%, including 91.4% of *E. coli*, 94.8% of *P. mirabilis*, 91.6% of *M. morganii* strains at MIC value 2 μg/mL) and some *P. aeruginosa* strains. In a study carried out Bailey et al. 2018, 428 members of the Enterobacteriaceae family were collected mostly from urine samples and 173 isolates of *P. aeruginosa* were collected mostly from respiratory specimens. The results showed that 91.8% of Enterobacteriaceae members were susceptible to Cef/taz and the species with the highest percentage of resistant were *E. cloacae* (15.1%) and *K. oxytoca* (12.1%). 87,9% of *P. aeruginosa* isolates were also susceptible. López-calleja et al. 2018, in an epidemiological study collection of 150 XDR isolates from Spanish hospitals reported that the activity of Cef/taz against the MDR and XDR isolates tested was 92.2%, it was only surpassed by colistin.

Cef/taz demonstrated no activity for one XDR *P. aeruginosa* isolate, where MIC value obtained was the highest (≥ 256 μg/mL). This phenomenon may be due to the plasmid-encoded ESBLs or MBLs that are emergent as a mechanisms of resistance in *P. aeruginosa* (López-calleja et al. 2018). Further studies would be needed for this strain, particularly at the molecular level to understand the development of resistance.

Four of the six *P. aeruginosa* isolates show a Cef/taz MIC value ≤ 2 μg/mL, it was show that resistance to Cef/taz by *P. aeruginosa* strains is still low. In other studies, like van Duin and Bonomo 2016, the MIC value obtained for this pathogen was higher (8 μg/mL). Regarding *P. aeruginosa* strains, Shortridge et al. 2017, concluded that 97.3% of 3737 non-repetitive strains were susceptible to this antimicrobial combination. Although some resistance mechanisms, such as overexpression of efflux pumps, have been demonstrably linked to the reduction of susceptibility to certain drugs, Cef/taz has been shown to be extremely stable in *P. aeruginosa* overproducing efflux pumps or AmpC β-lactamases and in strains lacking porin expression (Howland and Chesnel 2017). This ability of Cef/taz can explain the results obtained for strains *P. aeruginosa* in this study.

Thus, Cef/taz, *in vitro*, inhibits the growth of a high percentage of bacterial isolates from the clinical importance Enterobacteriaceae family and *P. aeruginosa.* As Kaye and Pogue 2015, Dandan et al. 2019 and López-calleja et al. 2018 reported, Cef/taz has greater activity than currently available cephalosporins, carbapenems, and piperacillin/tazobactam for the treatment of cUTIs, including pyelonephritis, caused mainly by Enterobacteriaceae members including ESBL-producing and MDR *P. aeruginosa* strains.

Cef/taz is a good choice for *P. aeruginosa* since it has the ability to withstand multiple resistance mechanisms (Montravers and Bassetti 2018). Although resistance is already detectable, Cef/taz has demonstrated active *in vitro* against strains of *P. aeruginosa* that are resistant to carbapenems, cephalosporins, fluoroquinolones, and/or aminoglycosides with a MIC that is 8- to 16-fold lower when compared to ceftazidime, imipenem, and ciprofloxacin (Ge et al. 2010; Sader et al. 2014a; Cluck et al. 2015; van Duin and Bonomo 2016; Giacobbe et al. 2018).

5.3 Conclusion

The great activity, *in vitro*, of Cef/taz revealed by this study and others suggests that this new combination of a cephalosporin with a well-known β-lactamases inhibitor

(ZERBAXA®) should be considered as an alternative antibacterial therapy to most of the MDR pathogens causing cUTI like *E. coli* and *K. pneumoniae*, particularly in high-risk patients. Normally, the common β-lactams are affected by overexpression of efflux pumps or AmpC, or by loss/alteration of OprD in *P. aeruginosa*, but this cephalosporin is less affected. Although in this study carbapenem-resistant Enterobacteriaceae isolates were not analyzed, Cef/taz is also a good alternative for these microorganisms. Nevertheless, cases of resistance to this new drug have already been reported, as demonstrated in this study, emphasizing the need to evaluate the susceptibility to Cef/taz in clinical Microbiology laboratories.

Further studies of Cef/taz in combination with other antibiotics are warranted to explore the activity against other resistant Gram-negative pathogens.

In short, the use of the correct antibiotics is crucial to decreasing the dissemination of resistant strains, both in the hospital setting and in the community, and consequently, to reduce mortality and morbidity rates as well as costs at the level of public health.

6 Chapter 3. Development of pedagogic materials for antibiotic resistance awareness

6.1 Introduction

Currently, public involvement in Health and Science content is needed. It is increasingly intended to contribute to a cultured society at the scientific level, capable of actively participating in and supporting the current research and innovation challenges. Despite the growing number of projects and initiatives aimed at society at large, the promotion of scientific knowledge still faces major challenges. It is up to the science communicators to understand how to reach the public and to identify strategies that increasingly arouse society's interest in themes of scientific culture.

This work was developed based on the participation in the "Projeto de Estímulo à Mobilidade de Ideias" (PEMI), that is one of the educational projects developed by the Association for World Innovation in Science and Health Education (AWISHE). This association has as its starting point to reach some of the Sustainable Development Goals of the WHO. These goals aim to develop a better and more sustainable world for all. They address the global challenges we face, including those related to poverty, inequality, climate, environmental degradation, prosperity, peace and justice. The different objectives interconnect, and not to leave anyone behind, it is important to reach each of them by 2030. Ensuring healthy lives and promoting the well-being at all ages is essential to sustainable development. So, currently, AWISHE focuses on five major goals:

- Enable training and awareness actions about Science and Health;
- Develop educational activities for children, youngsters and adults;
- Develop a permanent link with educational and cultural programs;
- Promote the development of national and international Collaborative Learning Communities;
- Promote access to information, educational opportunities, training and development.

The PEMI project is a pioneering project driven by the great mission of this Association: the awareness of Science and Health issues through Education. This project, to be implemented in schools of primary and secondary education, develops lectures of scientific character and practical activity for the sharing of knowledge, between the scientific community and the schools. To be able to address several scientific areas such as Microbiology, Anthropology, Medicine, Neurosciences, among others, PEMI is made up of

a versatile team of scientists, researchers, doctors and nurses. It is intended that the content transmitted is of interest to the young, school-aged, to easily capture their attention and so that it can be assimilated by all. In this way, it is hoped to train these young people as citizens who are part of a conscious and proactive community.

This project arises from the need to share information between the scientific community, and a group of students with an Alternative Curricular Course, at risk of dropping out of school, aiming at a social transformation. In this case, these students have no future expectations, requiring training for citizenship after compulsory schooling to become responsible, informed citizens with the capacity to intervene socially in subjects of scientific culture.

So far, PEMI was only implemented during the 2nd period of the 2018/2019 school year in the "Agrupamento de Escola de Oliveirinha", for 25 students with an Alternative Curricular Course. These students attend subjects of an obligatory nature, but the contents are approached in a less theoretical and more social way. One of these disciplines, "Saúde e Ambiente", is part of the Sciences, in which PEMI was integrated. To reach several scientific subjects, it was divided into different sessions.

This dissertation then contributed to one of the sessions, entitled "O (ir)resistível mundo dos antibióticos". With this session was intended the scientific dissemination of the worrisome and current global health problem - antimicrobial resistance. As mentioned in previous chapters, antimicrobial resistance is associated with the increase in mortality and morbidity rates, and it is estimated that by 2050 it will be the cause of around 10 million deaths, annually (WHO 2014; Potron et al. 2015; Marston et al. 2016; Montravers and Bassetti 2018; Koulenti et al. 2019).

This worrisome phenomenon is the result from the inappropriate and excessive use of antibiotics, in animal production, as prevention and growth factor, and at the clinical level as empirical therapy for various infectious diseases. An uninformed community, such as the students in question, may trigger the misuse of antibiotics. Usually, in these cases, the main precautions aimed at reducing the rate of antimicrobial resistance aren't considered at all. Thus, to counter this lack of information on the part of these young people, theoretical sessions were developed on the basic structure of disease-causing bacteria, the different types of antibiotic used to combat them and how their excessive use led to the development of

resistance. The discourse was adjusted to the target audience, stimulating a scientific lesson using the play activity, as shown in Figure 45, capturing the audience, to consolidate the knowledge acquired, without ever losing scientific objectivity and rigor.

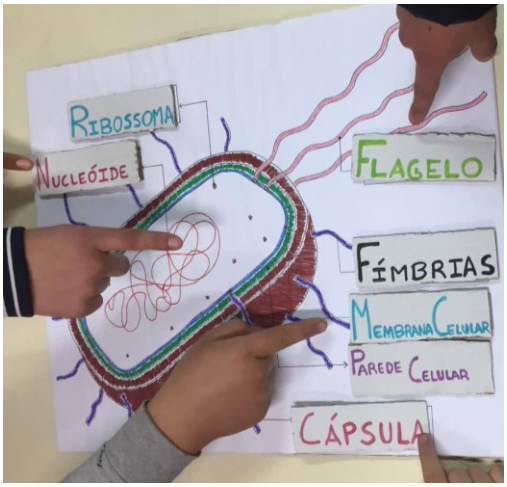


Figure 45 Illustrative example of some of the activities developed.

The practical activity shown in Figure 45 consisted in the definition of the structures that constitute the bacteria. An increased bacterial model was used, where the students had to make the correspondence of several structures previously introduced. Throughout this activity, the development of different classes of antibiotics has also been explained, as well as the development of resistance by the bacteria temporarily accompanied the development of new classes of antibiotics. The students were divided into groups to make the most of the contents to be understood. After identifying the different constituents of the bacteria, each one was related to its function. Subsequently, it was explained how each class of antibiotic can act in the different bacterial functions, to prevent them and thus, to kill the bacteria. At the same time, it has also been shown that when an antibiotic acts on a certain structure/mechanism of the bacteria, it can develop the ability to adapt and prevent the action of antibiotic - antimicrobial resistance.

6.2 Results and discussion

A multiple-choice questionnaire (APPENDIX 4) as a method of evaluating the knowledge acquired, was distributed at the end the session. The classification was positive in about 70% of the questionnaires, validating the application of the project and acceptance

by the students. However, among the 25 students, questionnaires with zero correct answers appeared. Considering the lack of interest at school level by this public, this method of evaluating doesn't have the desired impact. Alternative methods of evaluation, such as oral or practical activities are suggested so that it can show a greater receptiveness of the content addressed.

6.3 Conclusion

Evaluating the success of the first project activity, the PEMI team intends to expand this project to regular teaching classes, complementing the regular curriculum, covering other levels of education. In this way, PEMI will continue to promote the inclusion of both students with alternative courses and promoters who are part of it. With projects such as the PEMI, it is expected that citizens of the community at large will be sensitized to arouse their interest and to trigger conscious and appropriate opinions and decisions on scientific issues and, in this case, on antimicrobial resistance. We are building a better, more cultured and consequently healthier future for people around the world!

7 General conclusions

Currently, there is a dramatic increase in the proportion of bacterial pathogens resistant to multiple antimicrobial agents. On the one hand, the high antimicrobial resistance rates are directly related with the abuse and misuse of antimicrobial agents in patients and livestock that are unintentionally released into the environment. On the other hand, due to lack of development of new effective drugs by the pharmaceutical industries.

The results that are included in this thesis are of utmost importance, since they constitute a study carried out to analyze the epidemiology of strains resistant to colistin in hospital environment. Therefore, this study shows a great activity of the new drug – Cef/taz, which can be introduced in clinical practice for the treatment of cUTIs, that are one of the main HAI. So, this study reinforces the need to develop surveillance strategies and to implement specific control procedures to reduce the inappropriate use of antibiotics.

Additionally, this study helps to highlight the importance of scientific communication for the whole community, so that, together, we contribute to the reduction of antimicrobial resistance.

8 Looking Ahead

In the future, it will also be important to understand how the *mcr-1* gene develops resistance to colistin, as it is not yet known. The hypothesis put up until then is the relationship with zinc metal. There is an interaction between the catalytic domain of the enzyme MCR and the use of zinc as a food additive in the production of pigs and in their antimicrobial therapy, since this has already been observed for two other transferases enzymes that confer polymyxin resistance. Moreover, it is further known that *mcr-1* is often isolated of the soil and of the water, where also the zinc metal is found as contaminant. So, Sun et al. 2018 hypothesize that the emergence of MCR-like enzymes might be the result of the recruitment of zinc by these transferases in Enterobacteriaceae due to a selection coming from heavy-metal pressure. However, further research is needed to substantiate this hypothesis.

The emerging development of different variants of the *mcr* genes indicates that this type of resistance may represent a troubling challenge at the level of clinical therapies for public health. This highlights the urgent need to act at both the national and global levels to combat resistance to colistin.

Although antibiotics are a good resolution for the control of infectious diseases caused by MDR organisms, their use is currently compromised due to antimicrobial resistance, so the development of other alternatives is extremely necessary. Alternatives such as targeting quorum sensing systems, lectin inhibition, bacteriophage, immunotherapy, delivery of drugs, liposomes as drug targeting vehicles or even use of natural compounds must be explored as soon as possible.

Another major contribution to antimicrobial resistance is the lack of knowledge on the part of the whole general community. Development of projects such as PEMI, applied to a very diversified public, from children to the elderly, can become an asset in the fight against this great public health problem.

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10 Appendices

10.1 Appendix 1

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

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

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

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

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

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

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Introduction

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

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

Purpose

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

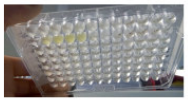
Methods

Collection of strains during 2017-2019

Culture plates

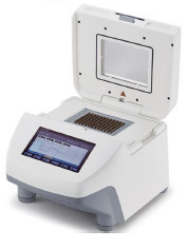
VITEK2®

Identification of 13 strains resistant to colistin



MICRONAUT-S

Confirmation of colistin resistance



Thermocycler CFX PCR

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

Results and conclusions

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

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10.2 Appendix 2

PEMI: Mobilidade de ideias no caminho da inclusão

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

O PEMI, a ser implementado em escolas do ensino básico e secundário, dinamiza palestras de carácter científico, como veículo de informação entre a comunidade científica e as escolas, que incluem atividades práticas hands-on para consolidar os temas abordados em cada sessão. Dentro das diferentes áreas que este projeto leva às escolas, são abordados temas de interesse aos jovens em idade escolar, com especial ênfase em comportamentos e ações que podem ser apreendidos e facilmente replicados, para que todos quantos os recebam passem a fazer parte de uma comunidade consciente e pró-ativa.

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

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

Numa primeira fase, o PEMI foi implementado, ao longo do 2º período do ano letivo 2018/2019, no Agrupamento de Escolas de Oliveirinha, numa turma de Percurso Curricular Alternativo, com 25 alunos. A estes alunos é pedido que frequentem disciplinas de carácter obrigatório, mas numa vertente mais social e menos teórica. No contexto das Ciências, uma

das disciplinas propostas é a disciplina “Saúde e Ambiente”, onde se integrou o PEMI, sensibilizando os alunos para a importância de evitar comportamentos de risco, e assumir ações que levem a uma boa qualidade de vida, individual e comunitária. No final de cada sessão, foi aplicado a todos os alunos um questionário de escolha múltipla, testando os tópicos essenciais abordados. Até à data, as classificações foram positivas em mais de 50% dos alunos envolvidos, validando aplicação do projeto e aceitação por parte dos alunos.
Para além da aplicação em turmas de ensino não regular como esta, o PEMI constitui também um complemento ao currículo regular. Considerando o potencial de crescimento e aplicabilidade, o PEMI vai continuar a promover a inclusão, tanto de alunos de diferentes contextos, como de promotores que dele vierem a fazer parte.

Palavras-chave: Sensibilização, Comunidade de Aprendizagem Colaborativa

PEMI: Mobilidade de ideias no caminho da inclusão

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

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

Palestras de carácter científico rigoroso e atividades práticas divertidas

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

Equipa multidisciplinar

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

Complemento ao ensino regular e estratégia para currículos alternativos

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

Comunidades de Aprendizagem Colaborativa

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

Resultados e perspetivas futuras

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

Para uma comunidade consciente e interventiva

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10.3 Appendix 3

O (ir)resistível mundo dos antibióticos

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

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

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

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

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

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

O (ir)resistível mundo dos antibióticos

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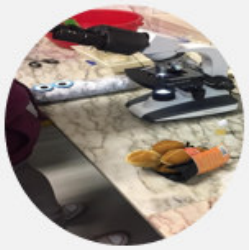
INTRODUÇÃO

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

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

BACTÉRIAS

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



VISUALIZAÇAO AO MICROSCÓPIO ÓTICO

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

LINHA DE TEMPO

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

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

RESULTADOS E DISCUSSÃO

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

CONCLUSÃO

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

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10.4 Appendix 4

QUESTÕES DE AVALIAÇÃO

1. Tendo em conta a **Coloração de Gram** as bactérias podem ser divididas em:
   a) Bactéria grande e bactéria pequena;
   b) Bactéria de Gram-negativo e bactéria de Gram-positivo;
   c) Bactéria roxa e bactéria azul;
   d) A coloração de Gram não permite dividir as bactérias.

2. A diferença entre bactérias de **Gram-negativo e Gram-positivo** é:
   a) A quantidade de peptidoglicano na parede celular;
   b) O número de núcleos na bactéria;
   c) Não há diferença nenhuma;
   d) A presença/ausência de flagelo.

3. O que é um antibiótico?
   a) Qualquer composto químico que cora as bactérias;
   b) Qualquer composto químico capaz de combater uma infeção num dado organismo causada por bactérias;
   c) Um composto químico que apenas pode ser usado na medicina veterinária;
   d) Composto usado no tratamento da gripe.

4. O antibiótico com maior importância histórica é
   a) Ainda não foi descoberto nenhum com importância histórica;
   b) Um β-lactâmico - a penicilina, descoberto em 2017 por Alexander Fleming;
   c) Uma cefalosporina de 3ºgeração descoberto em 1956 por Mack McCormick;
   d) Um β-lactâmico - a penicilina, descoberto em 1924 por Alexander Fleming;

5. A utilização excessiva e inapropriada de antibióticos leva…
   a) … a que estes fiquem cada vez mais eficazes;
   b) … ao combate de todas as bactérias patogénicas;
   c) … ao desenvolvimento de bactérias resistentes a esses antibióticos;
   d) … ao combate de todas as doenças.

6. Resistência bacteriana aos antibióticos é a capacidade de:
   a) A bactéria crescer e multiplicar-se na presença de um antibiótico;
   b) O antibiótico destruir/matar as bactérias;
   c) O paciente escolher qual o antibiótico que vai tomar;
   d) As bactérias destruírem o antibiótico.