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José Maria Serpa Laço

RIVERS AS ROUTES FOR THE SPREAD OF ANTIBIOTIC RESISTANCE: ROLE OF ANTHROPOGENIC PRESSURES AND RISKS TO HUMAN HEALTH

Dissertação no âmbito do Mestrado em Bioquímica orientada pela Professora Doutora Isabel da Silva Henriques e pela Doutora Marta Cristina Oliveira Martins Tacão e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia
da Universidade de Coimbra

Rivers as routes for the spread of antibiotic resistance: role of anthropogenic pressures and risks to human health

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Resumo

A resistência a antibióticos é um problema crescente a nível mundial associado ao uso abusivo de antibióticos tanto em humanos como animais. *Enterobacteriaceae* resistentes a cefalosporinas de 3ª geração geram grande preocupação, dado que são fármacos de primeira linha utilizados para tratar infeções causadas por estes agentes infeciosos. A resistência a este tipo de antibióticos é mediada por beta-lactamases de espectro alargado (ESBLs), sendo as CTX-M as mais prevalentes a nível mundial. O rio Lis é um rio com elevados níveis de poluição localizado na região centro de Portugal, sujeito a diversas pressões antropogénicas incluindo descargas ilegais de resíduos provenientes de suiniculturas. Este estudo tem como objetivos: 1) determinar a prevalência de *Enterobacteriaceae* resistentes a cefotaxima ao longo do rio Lis em dois anos; 2) determinar a afiliação filogenética, clonalidade e perfis de suscetibilidade a antibióticos de bactérias portadoras do gene *bla*$_{\text{CTX-M}}$ isoladas deste rio; e 3) caracterizar a diversidade de genes *bla*$_{\text{CTX-M,}}$ o seu contexto genómico e associação a elementos genéticos móveis. O rio Lis apresentou qualidade da água muito baixa nos 15 locais amostrados, tanto em 2018 como em 2019. Dados recolhidos em 2018 revelaram a presença de bactérias resistentes a cefotaxima em todos os locais, geralmente mais abundantes em locais com menor qualidade da água. Em 2019 foi observada a mesma tendência, mas não foi detetada qualquer bactéria resistente a cefotaxima em três dos locais amostrados. Dos 147 isolados resistentes à cefotaxima distribuídos por 9 géneros, em 68 (46%), pertencentes a 4 géneros, foi detetado o gene *bla*$_{\text{CTX-M}}$. A tipagem molecular mostrou a presença de isolados provavelmente clonais em diferentes locais e nos dois anos, sugerindo persistência das estirpes no rio e provavelmente fontes de poluição contínuas. Os isolados clonais recolhidos no mesmo local e no mesmo ano foram excluídos da restante análise. Os restantes 54 isolados pertenciam aos géneros *Escherichia* (n = 32), *Klebsiella* (n = 18), *Enterobacter* (n = 3) e *Citrobacter* (n = 1). Destes isolados, 79,6% mostraram perfis de multirresistência, com 2 isolados a revelarem baixa suscetibilidade ao imipenemo, um antibiótico utilizado em último recurso. Sete variantes diferentes de *bla*$_{\text{CTX-M}}$ foram encontradas, nomeadamente *bla*$_{\text{CTX-M-1,}}$ *bla*$_{\text{CTX-M-3,}}$ *bla*$_{\text{CTX-M-15,}}$ *bla*$_{\text{CTX-M-32,}}$ *bla*$_{\text{CTX-M-55,}}$ *bla*$_{\text{CTX-M-14,}}$ *bla*$_{\text{CTX-M-27}}$ e *bla*$_{\text{CTX-M-65}}$, sendo a *bla*$_{\text{CTX-M-15}}$ a mais prevalente (52,5% dos isolados), tal como descrito mundialmente. A variante *bla*$_{\text{CTX-M-65}}$ foi detetada pela primeira vez em Portugal. A IS*Ecp1* foi detetada na região a montante do gene para todas as variantes, e IS*903* ou orf477 estavam presentes na região a jusante do gene.

Os resultados deste estudo reforçam os graves problemas de poluição no rio Lis. Em locais com mais baixa qualidade da água foram registados os níveis mais elevados de resistência e prevalência do gene *bla*$_{\text{CTX-M}}$, sugerindo este gene como um marcador de poluição. O número elevado de variantes de *bla*$_{\text{CTX-M}}$ detetadas em *Enterobacteriacea*e, em contextos frequentemente descritos em isolados clínicos, sugere que a água do rio Lis representa um risco considerável para a saúde humana. Este risco deve ser avaliado em detalhe dado que a água deste rio é utilizada frequentemente para irrigação, pesca e atividades de lazer.

PALAVRAS-CHAVE: *bla*$_{\text{CTX-M,}}$ Poluição de rios, Resistência a antibióticos, β-lactamases, *Enterobacteriaceae*

Abstract

Antibiotic resistance is a growing worldwide problem derived from overuse of antibiotics in both humans and animals. *Enterobacteriaceae* resistant to 3rd generation cephalosporins are a great concern, as these are first-line drugs to treat infections caused by these common pathogens. Resistance to these antibiotics is mainly mediated by extended spectrum beta-lactamases (ESBLs), being CTX-M the most prevalent worldwide. The Lis river is a heavily polluted river in central Portugal, affected by several anthropogenic pressures including illegal waste discharges from piggeries. This study aims are: 1) to determine the prevalence of cefotaxime-resistant *Enterobacteriaceae* along the Lis river, in two years; 2) to determine the phylogenetic affiliation, clonality and antibiotic susceptibility profiles of *bla*$_{\text{CTX-M}}$ carriers isolated in Lis river; and 3) to characterize the diversity of *bla*$_{\text{CTX-M}}$ genes, their genomic context and association to mobile genetic elements. The Lis river presented a very low water quality in the 15 sampling sites, both in 2018 and 2019. Data from the 2018 campaign revealed the occurrence of cefotaxime-resistant bacteria in all sites, generally with higher rates in sites with lower water quality. In 2019 the same trend was observed but cefotaxime-resistant bacteria were not detected in three sites. From 147 cefotaxime-resistant isolates included in 9 genera, 68 (46%) carried the *bla*$_{\text{CTX-M}}$ gene and affiliated with 4 genera. Molecular typing revealed the presence of potentially clonal isolates in different sites and in the two years, suggesting survival of the strains in the river and probably continuous pollution inputs from the same sources. Clonal isolates collected in the same site and at the same time were excluded from further analysis. The remaining fifty-four isolates affiliated with *Escherichia* (n = 32), *Klebsiella* (n = 18), *Enterobacter* (n = 3) and *Citrobacter* (n = 1). From these, 79.6% showed a multiresistance profile with 2 isolates with reduced susceptibility to imipenem, a last-resort antibiotic. Seven different variants of *bla*$_{\text{CTX-M}}$ were found, namely *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-3}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-32}}$, *bla*$_{\text{CTX-M-55}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-27}}$ and *bla*$_{\text{CTX-M-65}}$, with *bla*$_{\text{CTX-M-15}}$ being the most prevalent (52.5% of the isolates), as reported worldwide. *bla*$_{\text{CTX-M-65}}$ was detected for the first time in Portugal. IS*Ecp1* was detected upstream the gene for all variants, and IS*903* or orf477 were present in the downstream region.

Results from this study reinforce the serious pollution problems in the Lis river. Sites with lower water quality also showed the highest resistance rates and the highest prevalence of the *bla*$_{\text{CTX-M}}$ gene, suggesting this gene as a pollution marker. The high number of *bla*$_{\text{CTX-M}}$ variants detected in *Enterobacteriacea*e, in contexts frequently reported in clinical isolates, suggest that the Lis river water may present relevant human health risks, which must be further assessed since this water is frequently used for irrigation, fishing and leisure activities.

KEYWORDS: bla$_{\text{CTX-M}}$, River pollution, Antibiotic resistance, β-lactamases, *Enterobacteriaceae*

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Abrreviations and Acronyms

ALA Alanine

AMC Amoxicillin/Clavulanic acid

AML Amoxicillin

ARB Antibiotic resistant bacteria

ARGs Antibiotic resistance genes

ATM Aztreonam

BLAST Basic local alignment search tool

BOX-PCR BOX elements – polymerase chain reaction

bla β-lactamase

bp base pair

C Chloramphenicol

CAZ Ceftazidime

CIP Ciprofloxacin

CLSI Clinical and Laboratory Standards Institute

CN Gentamicin

CTX Cefotaxime

dH_2O Distilled water

DNA Deoxyribonucleic acid

dNTPs nucleoside triphosphates

ECDC European Center for Disease Control

EDTA Ethylenediaminetetraacetic acid

EFSA European Food Safety Authority

EMA European Medicines agency

ERIC Enterobacterial repetitive intergenic consensus

ESBL Extended spectrum β-lactamases

EUCAST The European Committee on Antimicrobial Susceptibility Testing

FC Fecal coliforms

FEP Cefepime

FS Fecal streptococci

HGT Horizontal gene transfer

IPM Imipenem

IS insertion sequences

LB Luria-Bertani Broth

MDR multi-drug resistant

$MgCl_2$ Magnesium chloride

MH Mueller Hinton

mL milliliter

ORF Open reading frame

PBPs penicillin-binding-proteins

PCA Plate Count Agar

PCR Polymerase chain reaction

PG peptidoglycan

PRL Piperacillin

RNA Ribonucleic acid

rRNA Ribosomal RNA

SXT Trimethoprim/Sulfamethoxazole

TAE Tris-Acetato EDTA

TC Total coliforms

TE Tetracycline

TE Tris-EDTA

TIC Ticarcillin

TIM Ticarcillin/Clavulanic acid

TZP Piperacillin/Tazobactam

UN United Nations

UTIs Urinary tract infections

WFD Water Framework Directive

WHO World Health Organization

WWTPs wastewater treatment plants

XLD Xylose-lysine-desoxycholate

µL Micro liter

Introduction

1. Antibiotics

Over the course of mankind, many scientists observed microbial antagonism, but not many thought it could be caused by a certain compound. In 1875, John Tyndall hypothesized that limited oxygen conditions in the presence of *Penicillium* inhibited bacterial growth (Tyndall, 1876). By the end of the 19th century, antibiosis was the term used to define this microbial antagonism (Vuillemin, 1889). In 1897, Ernest Duschesne found antagonism between *Penicillium* and *Escherichia coli* in vitro. He also tested this discovery in guinea pigs, by co-injecting a normally lethal dose of *Salmonella Typhi*, the causative agent of typhoid fever, with *Penicillium glaucum*, and observed that the animals did not get typhoid (Duschesne, 1897). Later, in 1920, Andre Gratia and Sara Dath published their observations on the bacteriolysis phenomena with relations to the fungus *Penicillium* (Gratia and Dath, 1924). Therefore, Alexander Fleming was not the first to observe the fungal-bacterial relation, but was the first to study penicillin, back in 1928, although it only began being commercialized in 1940 (Fleming, 1929).

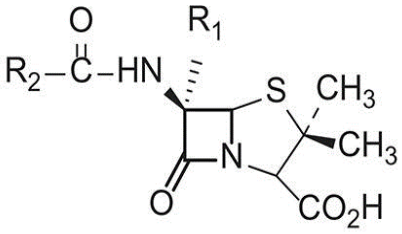
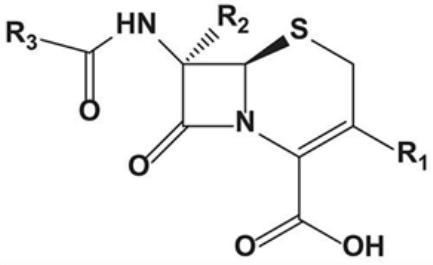
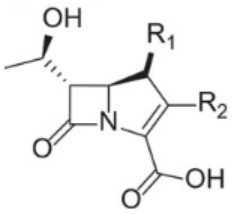
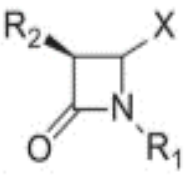
Antibiotics were first described as compounds produced by microorganisms that could inhibit bacterial growth (Waksman & Woodruff 1940), even though currently an antibiotic is any medicine used to treat bacterial infections. Since the discovery of the first antibiotic, many different antibiotics have emerged, and their usage grew exponentially over the years. This "wonder drug" saved millions of lives, but the growing resistance to the drug may lower its effectiveness. There are many different classes of antibiotics, but among them the β-lactams are of particular relevance, since they are the most used group of antibiotics worldwide (WHO, 2018).

Antibiotics act against bacteria by interfering with essential processes to their multiplication and survival. There are five main mechanisms: cytoplasmic membrane inhibition (e.g. polymyxins), protein synthesis inhibition (e.g. chloramphenicol, glycosamides, macrolides, aminoglycosides and tetracycline), nucleic acid synthesis inhibitors (e.g. quinolones, nitroimidazoles and rifampicin), folic acid synthesis inhibitors (e.g. sulphonamides and trimethoprim) and cell wall synthesis inhibition, as this last one is the mechanism used by β-lactams (Donowitz and Mandell, 1988).

1.1 β-lactam antibiotics

The β-lactams are divided in several sub-classes, among the main ones we find penicillins, carbapenems, monobactams and cephalosporins (table 1). Their common characteristic is the β-lactam ring structure, which is constituted by 3 carbon atoms and one nitrogen atom with a substituent (Nightingale et al., 1975).

Table 1. β-lactams classification and examples (Bush & Bradford, 2016)

Class	Subclass	Example	Base molecule
Penicillins	Penicillins	Penicillin	
	Aminopenicillin	Amoxicillin, Ampicillin	
	Ureidopenicillin	Piperacillin, Ticarcillin	
	Carboxypenicillin	Cloxacillin, Oxacillin	
	Penicillin resistant to penicillinases	Meciliname	
	Aminopenicillin	-	
Cephalosporins	1st generation	Cafalotin, Cefazolin	
	2nd generation	Cefuroxime	
	3rd generation	Cefotaxime, Ceftazidime	
	4th generation	Cefepime	
Carbapenems	-	Meropenem, Imipenem	
Monobactams	-	Aztreonam	

The β-lactams act by inhibiting the biosynthesis of the bacterial cell-wall (Figure 1), since they interrupt the transpeptidation process that links the peptidoglycan (PG) components of the cell wall (Donowitz and Mandell, 1988). The cell wall is an essential polysaccharide structure present in most bacteria that prevents osmotic rupture of the cytoplasmic membrane. It is formed by the PG, which consists of glycan chains with attached peptides used to crosslink adjacent glycans to form a matrix structure (Cho et al., 2014). β-lactams inactivate enzymes called penicillin-binding-proteins (PBPs). These enzymes bind to the D-Ala-D-Ala at the end of muropeptides (PG precursors) to crosslink the PG. However, the β-lactam ring is similar to the D-Ala-D-Ala, and so PBPs end up binding to the β-lactam and start using them in the cell wall synthesis. This action comes with a price, as PBPs are inactivated, leading to an accumulation of cell wall precursor units that activate the cell wall autocatalytic system, leading to cell lysis (Zapun et al., 2008). There are several types of PBPs encoded by bacteria that are involved in PG assembly (Sauvage et al., 2008). β-lactams have different affinities for different types of PBPs, the specific PBP bound has different effects on bacteria. For example, some PBPs such as PBP 1A, 1BS, 2 and 3, when inactivated lead to bacterial cell death. On the other hand, inactivating PBP4, 5, and 6, which are not essential for bacterial viability, is not lethal to the bacteria (Donowitz and Mandell, 1988).

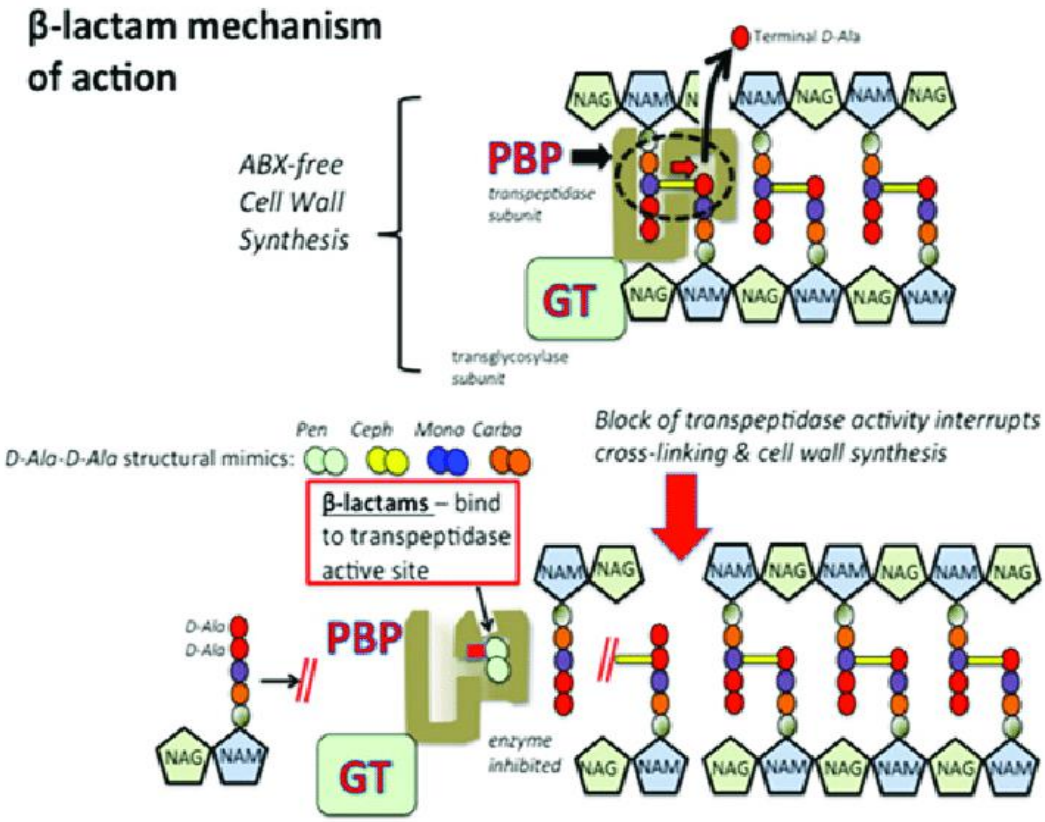


Figure 1. Schematic representation of the β-lactam mechanism of action (Retrieved from http://tmedweb.tulane.edu/pharmwiki/lib/exe/detail.php/cellwall_bls.png?id=antibiotic_pharmacology)

In clinical usage, β-lactams are often combined with β-lactamase inhibitors to improve effectiveness of the drug. Common examples are clavulanic acid, sulbactam and tazobactam. These compounds have the ability to inhibit diverse types of plasmid-mediated β-lactamases (Payne et al., 1994). However, the effectiveness of a certain combination is influenced by several factors, such as the type of β-lactamase produced by the bacteria (Thomson et al., 1990).

Cephalosporins were first introduced to clinical use in the 1960s and since then they have been used at a worldwide scale. In comparison to other agents, they show low rates of drug-associated toxicity as well as favorable pharmacokinetic (how a body processes a drug) profiles (Nightingale et al., 1975). Currently, cephalosporins, mainly 3rd generation cephalosporins, are very relevant as therapeutic agents, as they are the first line drugs used to treat infections, including pneumonia, meningitis and gonorrhea (Bronson & Barrett, 2012). The prototypic cephalosporin (cephalosporin C) is produced by a fungus called *Acremonium chrysogenum* (formerly *Cephalosporium acremonium*). Cephalosporins are divided in generations, based on their spectrum of activity. Essentially, 1st generation cephalosporins are effective against aerobic Gram-positive cocci, 2nd generation have little effect on Gram-positive and are more reliable on Gram-negative bacteria. The 3rd generation cephalosporins have a broad spectrum of activity against Gram-negative bacteria but are not very effective on Gram-positive. More recent, the 4th generation cephalosporins have a greater effect than the former ones on both Gram-positive and negative organisms, like cefepime. Differences between these generations rely on modifications of the cephalosporin C molecule. Variations can be achieved by substitutions of the side chains of the molecule R^1 and R^2. To increase compound stability against β-lactamases, usually the substitution is made ate the R^1 site (as seen in table 1). The change provides greater activity and a broader antimicrobial spectrum (examples are cefotaxime or ceftazidime) (Marshall & Blair, 1999)

3rd generation cephalosporin consumption in the EU in animals and humans is represented in figure 2 (ECDC/EFSA/EMA report, 2017). In Portugal as in other countries these antibiotics are preferentially used in human medicine, although relevant amounts are still used in veterinary or animal production.

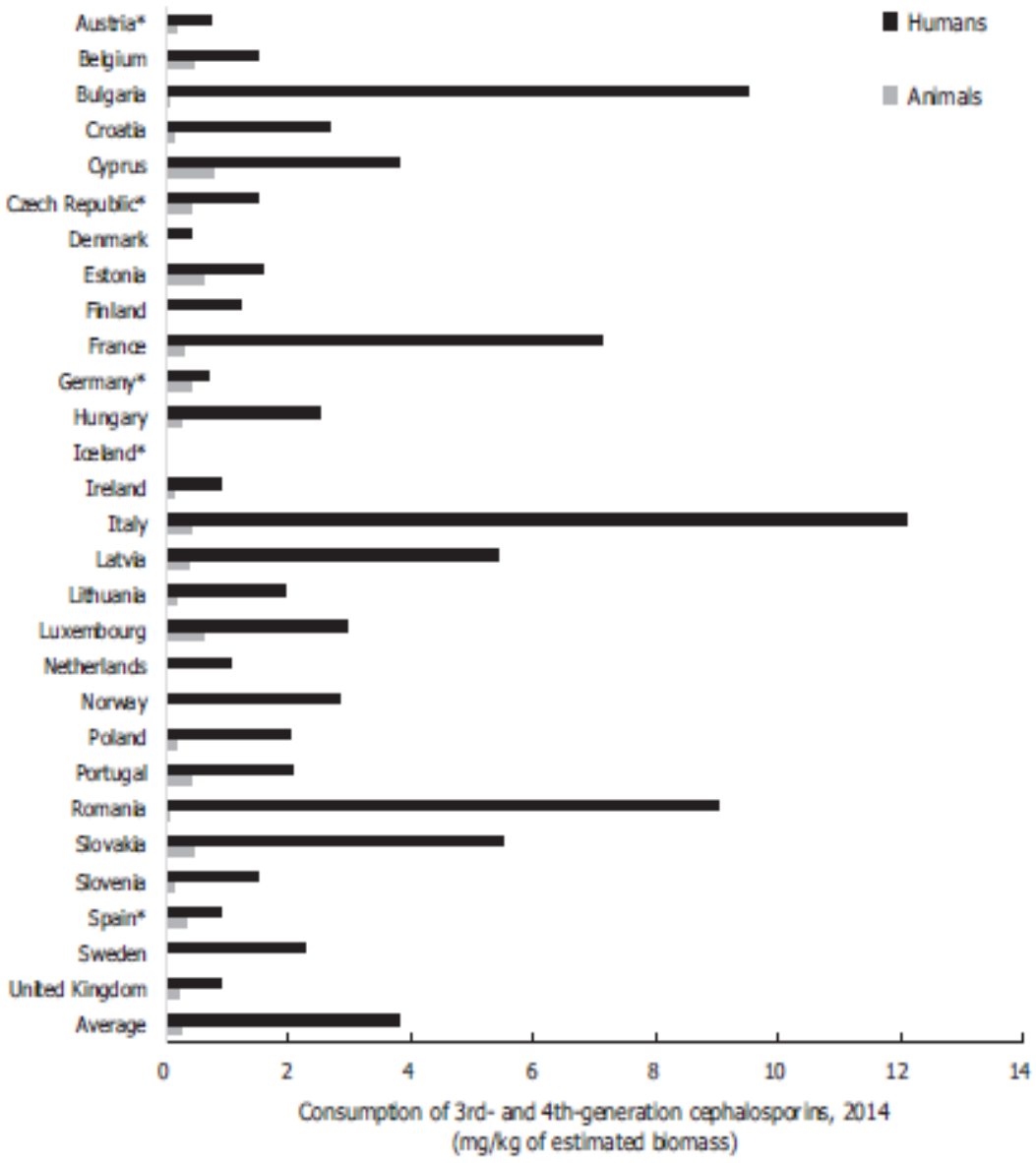


Figure 2. Consumption of 3rd and 4th generation cephalosporins in Europe (data retrieved from ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals, 2017)

2. Antibiotic resistance

A report from 2014 from the WHO (world health organization) revealed that antimicrobial resistance is set to be the major cause of death by disease by the year 2050, with an estimated 10 million deaths worldwide (figure 3), far more than the 8.2 million deaths per year attributed to cancer, the current major cause of death worldwide (O'Neill, 2014).

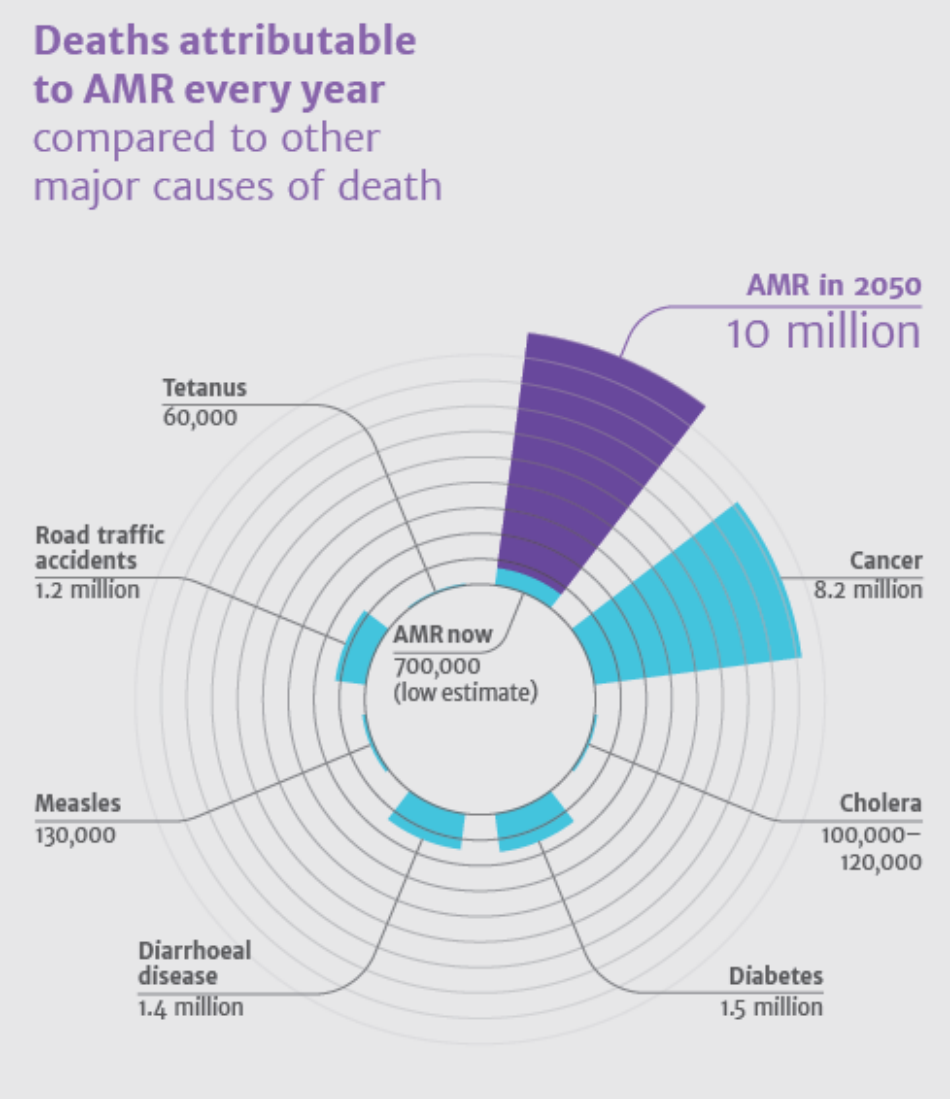


Figure 3. Major causes of death by year, in comparison to the current deaths attributed to antimicrobial resistance and the expected number for the same reason in 2050 (O'Neill, 2014)

Antibiotic resistance is a natural characteristic of bacteria and its increase and spread are inevitable results of antibiotic usage. However, this tendency may rise in time, as a result of abusive use (antibiotic usage to prevent infections may not be needed), insufficient use (when patients don't finish their treatment) or inadequate use (when the chosen antibiotic is incapable

of treating the infection due to bad diagnosis or for prophylaxis in animals, and even to promote animal growth, even though this last part isn't allowed in Europe) (Leung et al., 2011). Acquired resistance can appear as a result of two main mechanisms: mutation and/or horizontal gene transfer (HGT) (Martinez & Baquero, 2000). Mutations can occur spontaneously in the bacterial genome, resulting in the translation of altered proteins. Those new proteins can represent an advantage to that bacteria, and in the right conditions it will prevail at the expense of others. Mutations can be useful to survive the antibiotic effect and in the right media conditions (antibiotics present in the environment, for example), those bacteria will grow and develop. On the other hand, HGT occurs when a bacteria acquires new genes. This can occur by various mechanisms, such as: transformation (bacteria incorporate exogenous DNA in their genome), transduction (mediated by bacteriophages that carry DNA from other bacteria) or conjugation (when bacteria share plasmids or conjugative transposons). Antibiotic resistance genes (ARGs) can be present in plasmids, transposons and/or integrons. In addition, there are also intrinsically resistant bacteria. This is a trait often found in the genome of bacterial species and it does not correlate with antibiotic selective pressure or HGT. A good example of intrinsic resistance is the multi-drug resistant (MDR) Gram-negative bacteria, as their outer membrane makes them impermeable to many molecules and have multiple MDR efflux pumps that are effective in reducing intracellular concentrations of antibiotics. Intrinsic resistance is a common factor to all strains of given species (Nikaido, 1994). Examples include *Pseudomonas aeruginosa*, resistant to aminoglycosides, quinolones and β-lactams (Hancock & Speert, 2000) and *Kluyvera* spp. with the CTX-M-like β-lactamase (Cantón et al., 2012).

When exposed to β-lactams, bacteria have four major pathways to avoid their bactericidal effects:

1. Expression of altered and mutated PBPs (Fedarovich et al., 2012);
2. Absence or low expression of outer membrane proteins, only in Gram-negative bacteria (Delcour, 2009);
3. Overexpression of efflux pumps. These pumps are responsible for ejecting substances (including antibiotics) into the extracellular media, and its overexpression leads to less antibiotic concentration in the cell. Even though this mechanism is more effective against tetracyclines, it has been developing to provide resistance to β-lactams as well (Li & Nikaido, 2009);
4. Finally, is the production of enzymes called β-lactamases, capable of breaking the β-lactam ring and thus inactivating the antibiotic before reaching he PBP target. It's the most common mechanism in *Enterobacteriaceae* (Massova & Mobashery, 1997).

Resistance by β-lactamase production in Gram-negative bacteria can occur in many ways, but it is often linked with plasmid mediated extended spectrum beta-lactamases (ESBL), although it can also be related to other factors, such as chromosomal expression (Pitout, 2010)

β-lactamases received their nomenclature based on the name of the patient they were found (TEM: Greek patient named Temoniera), according to their substrate (OXA: active against oxacillin), from the hospital where they were found (MIR: Miriam hospital) or according to their biochemical properties (CTX-M: more activity against cefotaxime and first isolated in Munich) (Jacoby, 2006). As for classification, there are generally two main systems in use. The first one is the Ambler molecular classification, based on the conserved motifs and protein sequence, then dividing enzymes in four classes, A to D, even though Ambler only specified two classes initially (Ambler, 1980). All four classes have different specifications, class A are the active-site serine β-lactamases; class B the metallo-β-lactamases that require a bivalent metal ion (usually Zn^{2+}); class C, also known as AmpC β-lactamases, are also serine β-lactamases but have low similarity to the class A; finally, class D are also serine β-lactamases, but differ from both class A and C in their nucleotide sequences. They are also referred as OXA β-lactamases (Hall & Barlow, 2005). On the other hand, there is the Bush-Jacoby-Medeiros classification, which groups different β-lactamases according to their substrate and inhibitor profiles, as it correlates β-lactamase phenotype in clinical isolates. It's divided in three groups, group 1, 2 and 3 (Bush & Jacoby, 2010).

2.1 Extended spectrum β-lactamases (ESBLs)

Resistance to 3rd generation cephalosporins is mediated mainly by extended spectrum β-lactamases, or ESBLs, that confer resistance to all β-lactams, except to carbapenems and cephamycins (Nordmann et al., 2012). ESBLs are capable of hydrolysing and inactivate 3rd generation cephalosporins (like cefotaxime or ceftazidime) and are currently amongst the most globally dispersed ARGs (Chong et al., 2018). As mentioned before, inhibition of ESBLs can be mediated by clavulanic acid and tazobactam. First identified in 1983 (Knothe et al., 1983), these enzymes are now disseminated worldwide, mainly in the *Enterobacteriaceae* family, showing a significant growth in every WHO geographic region (Mondal et al., 2019; Ojer-Usoz et al., 2014). That growth is bigger when observing only developed countries and its upward trend is more significant in Europe (Bevan et al., 2017). ESBLs are very diverse, with CTX-M, TEM and SHV types being the most successful in terms of dissemination all over the globe (Bevan et al., 2017). Two main evolution strategies originated these enzymes, and their diversity and acquisition is related with: selection of mutants from TEM- and SHV-type β-lactamases, who have expanded substrate activity, and the capture of genes from the environment, that encode enzymes that have natural ESBL activity (Mondal et al., 2019). Among the former ones, CTX-M type β-lactamases are a huge success in terms of antibiotic resistance dispersion, being currently the most prevalent ESBLs worldwide (Bevan et al., 2017).

2.2 CTX-M enzymes

CTX-M enzymes are cephalosporinases that contain serine in their active site and belong to Amber class A. The gene that codes for these enzymes is the *bla*$_{CTX-M}$ and is usually associated to plasmids and insertion sequences (IS), as well as mechanisms of co-resistance (Toleman et al., 2006). Even though the majority of β-lactamases have an unclear origin, the ancestor of the *bla*$_{CTX-M}$ gene (Figure 4) is known to be present in the genome of species of the genus *Kluyvera*, belonging to the *Enterobacteriaceae* family (Sarria et al., 2001). The genes passed on to other bacteria by HGT.

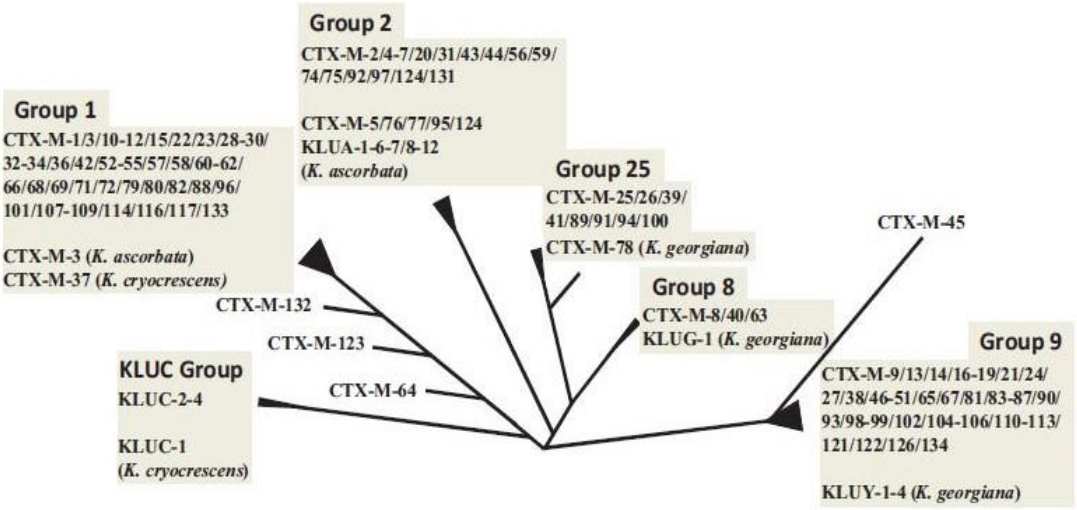


Figure 4. CTX-M enzyme variants (adapted from D'Andrea et al., 2013)

There are at least 217 different variants of CTX-M enzymes detected by 04/05/2020 (ftp://ftp.ncbi.nlm.nih.gov/pathogen/Antimicrobial_resistance/). In figure 3 we can see the diversity of CTX-M enzymes, as they are divided in six groups, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25, and KLU-like (genes that have been detected in the chromosome of strains of *Kluyvera* spp.). Members of the same group have >94% amino acid identity and ≤90% identity across different groups. Worldwide, the most common variants are CTX-M-14 and CTX-M-15, followed by CTX-M-1, CTX-M-2 and CTX-M-3 (Levy, 2002; Bevan et al., 2017).

3. Pollution in aquatic systems

Aquatic systems are heavily polluted at a global scale. Natural processes, such as precipitation, erosion, anthropogenic influences (urban, industrial and agriculture) and intensive exploration of hydric resources, determine surface water quality of a region (Carpenter et al., 1998). Rivers are amongst the most intensively human influenced ecosystems in the world (Tejerina-Garro et al., 2005). In addition, the human population is growing, being expected to reach 8.5 billion by 2030 (UN, 2019), and consequently, industrial and agricultural activities will increase as well. Climate change also threatens to affect the global hydrologic cycle (UN-Water, 2010). The main organic pollutants that are loaded into rivers worldwide are from industrial and domestic discharges, as well as intensive livestock farms (Meybeck, 2003) and it is estimated that more than one-third of the Earth's accessible freshwater is used for agricultural, industrial, and domestic purposes, activities that lead to water contamination (Anderson et al., 2006; Loos et al., 2009). Main pollutants in water include gasoline additives, surfactants, endocrine disruptors, pharmaceuticals and personal care products (Picó & Barceló, 2015). If not treated, the accumulation of these pollutants (natural and anthropogenic) in water can lead to serious contamination problems with long-term effects on aquatic life and human health. This enables a stimulation of microbial growth, which leads to an oxygen depletion and disturb in all river ecosystem (Sirota et al., 2013). Different pollution sources are found along a river, but their impact extends to downstream of the river, as pollutants (including bacteria) are transported along the river (Nelson & Murray, 2008).

Non-metabolized antibiotics enter the environment through animal manure and human wastes (Berendonk et al., 2015) and their future depends on the specific biological and physico-chemical properties of each antimicrobial compound. With this in mind, many antibiotics persist in all kind of environments, including water (Gothwal & Shashidhar, 2015).

In a 2018 report by the European Environment Agency (Kristensen et al., 2018), it was referred that only 40% of surface waters (rivers, lakes and transitional and coastal waters) revealed to be in a good ecological status, and only 38% were in good chemical status. Mercury was found to be the main chemical pollutant, as omitting its effects showed that only 3% of surface waters would have poor chemical status. In order to monitor water bodies, the European union (EU) countries must follow the Water Framework Directive (WFD, https://ec.europa.eu/environment/water/water-framework/index_en.html). Member states have to protect, enhance water quality, and restore all water bodies and ensure a balance between abstraction and recharge, with the aim to achieve good status (chemical and ecological).

3.1 Antibiotic resistance in aquatic systems

Antibiotic resistant bacteria (ARB) and ARGs are a growing concern in surface waters that result from selective pressures caused by antibiotics (and other pollutants) on the microbial community of a particular environment, leading to serious ecological risk (Berendonk et al., 2015). ARB and ARGs are naturally present in ecosystems but can also enter aquatic environments through discharges of untreated wastewater from different sources (agriculture, industry, hospitals for example), as well as wastewater collection systems and can even be transferred to soils after. The water is also fundamental for agriculture and livestock, meaning that using that water can have serious impact on both activities (Baquero et al., 2008; Lupo et al., 2012; Tacão et al., 2012). In a 2008 report (Baquero et al., 2008), these authors pointed out the need to prevent a mixture of human and animal bacteria with environmental organisms, as this mixture can promote antibiotic resistance. They also defined four genetic reactors that gather the ideal conditions for antibiotic resistance evolution to occur and that present a high biological connectivity, generation of variation, and presence of specific selectors. These four reactors can be seen schematically in figure 5.

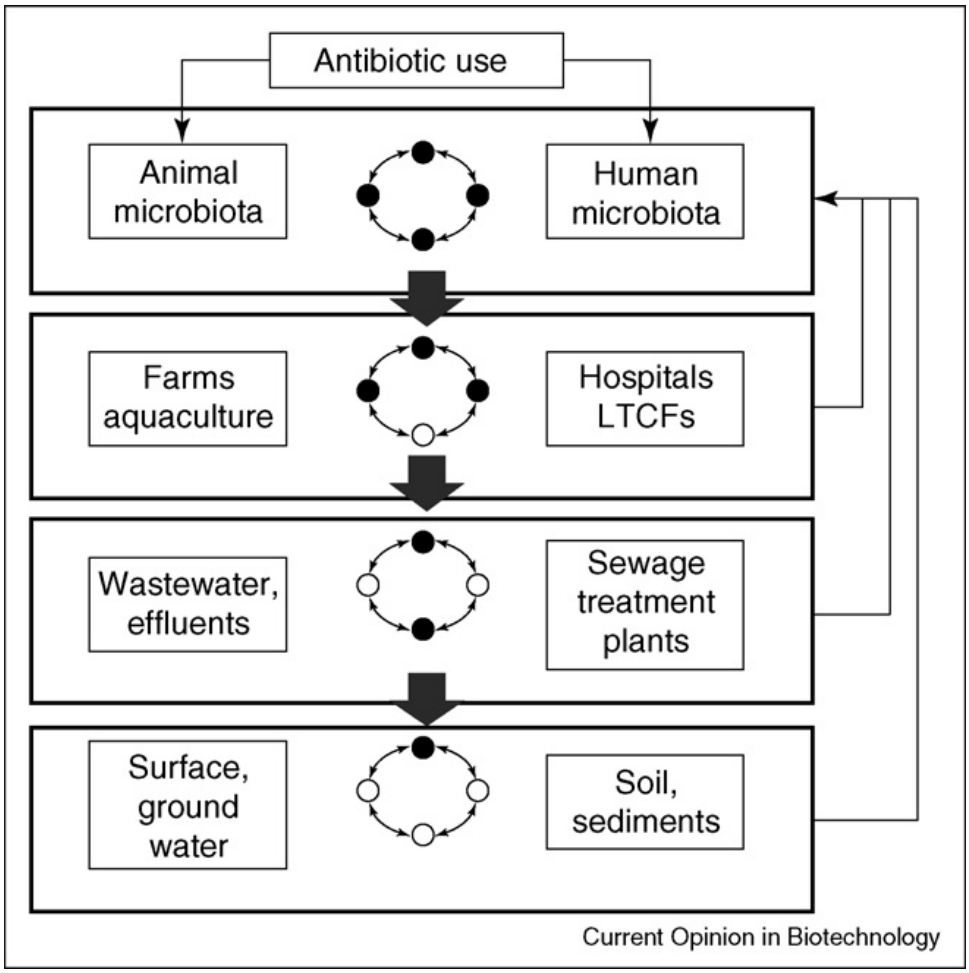


Figure 5. The four genetic reactors of ARG dissemination (Baquero et al., 2008).

As ARGs are distributed through aquatic systems worldwide, including Portugal (Sharma et al., 2016; Tacão et al., 2012; Teixeira et al., 2020), it is becoming more and more important to study the impact that they have in our lives. Besides human health, wildlife can also be negatively affected by this growing problem, as its presence may disturb natural environments in a negative way (Ashbolt et al., 2013; Martinez, 2009). As such, ARB and ARGs are being considered as a major public health concern.

Moreover, wastewater treatment plants (WWTPs) are considered as hotspots for ARB and ARGs accumulation and dissemination. They contain sub therapeutic concentrations of antibiotics and other co-selective agents consumed by humans and animals (Rizzo et al., 2013) that are then released in the environment (Karkman et al., 2019). Furthermore, the release of WWTP discharges contributes for the environmental dissemination of ARB and ARGs, as the bacteria surviving the treatment will interact with environmental bacteria (Bengtsson-Palme et al., 2018). Consequently, the environment itself is a hotspot and route of dissemination for antibiotic resistance (Wright, 2010).

4. *Enterobacteriaceae* and their relevance

Enterobacteriaceae are a family of Gram-negative bacteria that are among the most abundant commensal organisms in humans and other animals. They are also characterized as facultative anaerobes and non-sporing bacilli that ferment glucose and other sugars, reduce nitrate to nitrite and produce catalase but do not produce oxidase (except *Plesiomonas*). Members of this family are regularly introduced as enteric, because they mostly inhabit the lower gastrointestinal tract of a wide variety of animals. However, being enteric doesn´t imply that the bacteria in question belongs to the *Enterobacteriaceae* family, as other bacteria also colonize the same habitat, such as *Vibrio* spp. (also referred as enteric) (Donnenberg, 2014). In figure 6 a representation of some *Escherichia* and *Klebsiella* isolates in m-FC agar can be seen.

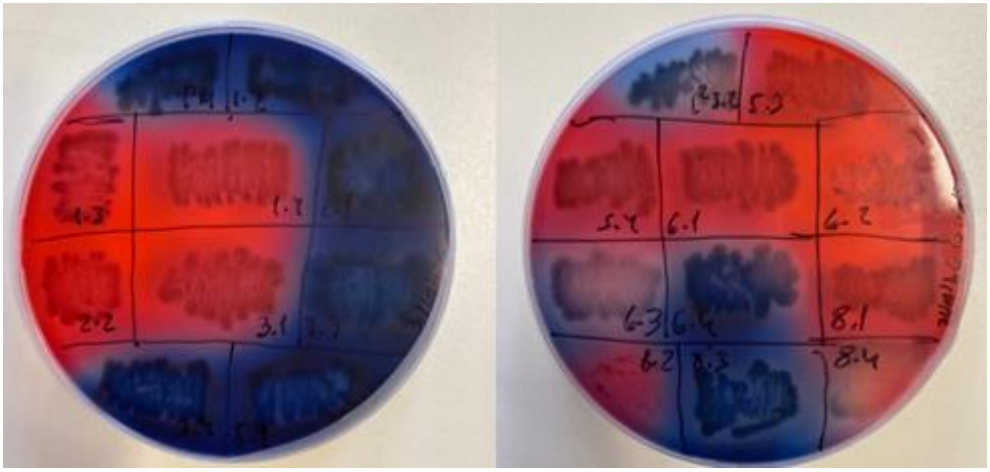


Figure 6. *Enterobacteriaceae* colonies in m-FC agar with representatives from *Escherichia spp*. and *Klebsiella spp*.(m-FC agar plates are from this study).

Besides being commensal, these bacteria can also be saprophytic or pathogenic. In fact, they are the main cause of infections in humans of all ages (Sedgley & Samaranayake, 1994), causing mainly urinary tract infections (UTIs), respiratory tract infections, bloodstream infections, hospital and healthcare associated pneumonias, as well as some intra-abdominal infections (Paterson, 2006). For example, UTIs are frequently linked with *Escherichia coli* and *Klebsiella* spp. whereas *Enterobacter* spp. are linked to pneumonia (Paterson, 2006). Moreover, *Enterobacteriaceae* in general can cause bloodstream infections, and are currently among the predominant bacteria concerning the antimicrobial resistance problem (Teklu et al., 2019). In fact, the world health organization ranked *Enterobacteriaceae* resistant to carbapenems and/or 3rd generation cephalosporins as critical for the development of new antibiotics (WHO, 2017). Their ubiquity, alongside with a natural ability to acquire mobile genetic elements means that we, humans, are repeatedly being exposed to new strains of bacteria with a wide variety of gene combinations, from the water we drink to animate or inanimate sources everywhere (Grundmann et al., 2010).

5. The Lis river case

The Lis river basin is located in the central region of Portugal, with a total area of approximately 850 km^2 and is one of the most important natural resources of the Leiria region (figure 7). Through a hydrological year, the Lis river and its affluents receive high levels of precipitation during the rainy months, and almost none in the dry season. This river is subjected to successive discharges of effluents over its course, which result in a decrease in water quality (Vieira et al., 2012). There are currently three WWTPs in the region (Olhalvas, Coimbrão and Vieira) and all of them discharge in the Lis river.

There are around 400,000 pigs in the Leiria region that produce waste equivalent to 1.2 million people (Vieira et al., 2012). Studies revealed that piggery wastewater discharges have a great impact on water quality in the region, also revealing that the values of fecal coliforms (FC) are above allowable limits for bathing waters, with special concern in the Vieira de Leiria beach, the Lis river mouth (Vieira et al., 2012, 2013). Total coliforms (TC), FC and fecal streptococci (FS) are indicators of water quality, and FC are a subgroup of TC that are mostly present in the intestines of warm-blooded animals, including humans.

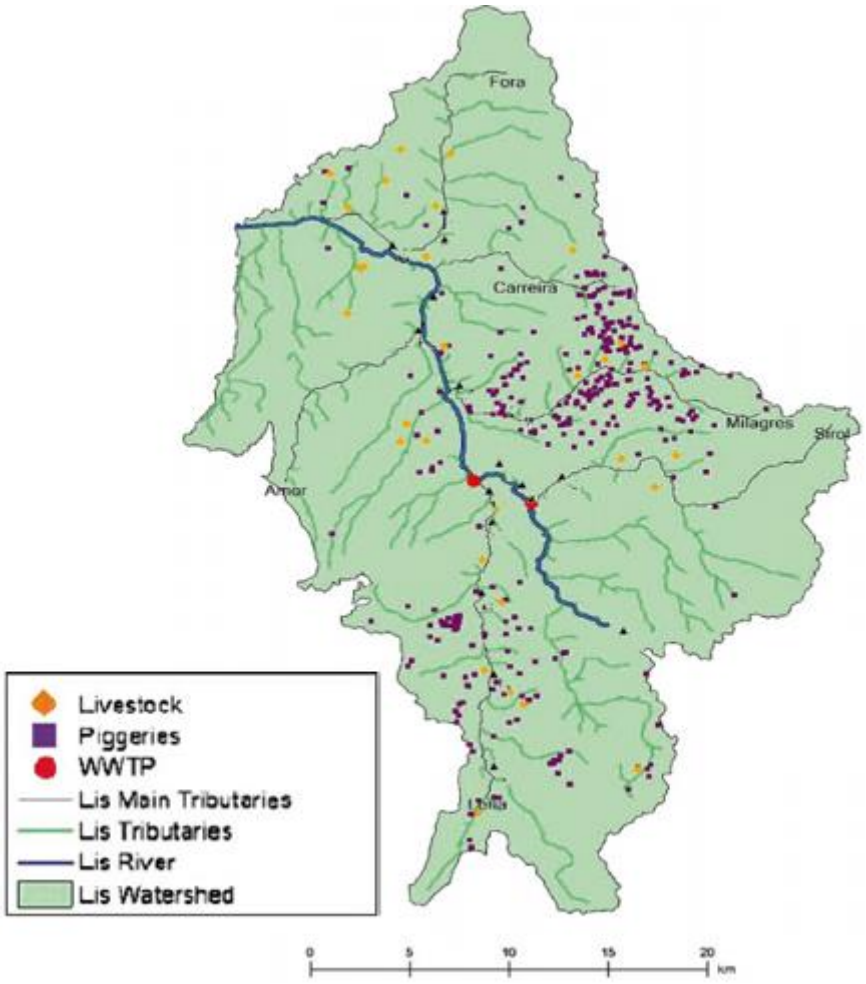


Figure 7. Lis river and its tributaries, along with indication of piggeries, livestock and two WWTPs (adapted from Vieira et al., 2012)

Therefore, piggery untreated wastewater discharges are a huge problem affecting Lis river and its affluents, mainly the affluent Ribeira dos Milagres, where most of the piggeries are located (as seen in figure 7). Furthermore, domestic effluents, untreated sewage and agriculture also carry to the river degradable organics, nutrients and pathogenic organisms (Vieira et al., 2012). In addition, there are also reports of different contaminants in the river, such as metals, mainly Zn and Mn, associated to anthropogenic sources (Vieira et al., 2009). Pharmaceutical contamination was also evaluated in a previous study (Paíga et al., 2016), and showed that ibuprofen, as well as antibiotics sulfamethoxazole, clarithromycin and azithromycin represented a potential risk for fish and algae. Moreover, the first study regarding antibiotic resistance in the Lis river was published this year, focused on carbapenem-resistant *Enterobacteriaceae* (Teixeira et al., 2020). In this study, several clinically relevant *Enterobacteriaceae* resistant to carbapenems, as well as to other antibiotics (including cefotaxime, cefepime and ceftazidime) were found in the Lis river, carrying ARGs clinically important and that have been associated to hospital outbreaks worldwide, including Portugal (Teixeira et al., 2020).

Objectives

Antibiotic resistance is a growing public health concern. Infections caused by *Enterobacteriaceae* resistant to 3rd generation cephalosporins are becoming more common worldwide, hence these bacteria have been classified as one critical group to develop new antibiotics.

Bacteria in the environment are frequently exposed to selective pressures from all types of sources that promote transfer of ARGs. Anthropogenic pressures, mainly because of overuse of antibiotics in both humans and animals, promote the arising of resistant bacteria as well as of new resistance genes in natural environments. As such, the environment plays a major role in spreading ARGs and ARB.

With the contaminants load that many rivers present worldwide, these environmental compartments became hotspots for ARGs and ARB, affecting human populations as well as the wildlife. In Portugal, the Lis river is an example of a highly polluted aquatic system mainly due to illegal discharges of animal production waste.

As such, studying the antibiotic resistance problem in the Lis river is crucial, as recently demonstrated by the presence of carbapenem-resistant bacteria in this river water (Teixeira et al. 2020), which alarms for the need to evaluate the presence of 3rd generation cephalosporins-resistant bacteria as well, since they are first line drugs to treat Gram-negative infections.

With this in mind, this study primarily intends:

- To determine the prevalence of cefotaxime-resistant *Enterobacteriaceae* along the Lis river, in two years;
- To determine the phylogenetic affiliation, clonality and antibiotic susceptibility profiles of $bla_{\text{CTX-M}}$ carriers isolated in Lis river;
- To characterize the diversity of $bla_{\text{CTX-M}}$ genes, their genomic context and association to mobile genetic elements.

1. Culture media

Several different culture media were used in this study, as shown in table 2. m-FC agar is a selective and differential culture medium able to inhibit bacterial growth except of fecal coliforms. Note that from the point 5 in this section onwards, the isolates were cultivated in Chromocult instead of m-FC agar. Chromocult contains Tergitol® 7, that inhibits the growth of gram-positive bacteria, as well as some Gram-negative, except for coliform bacteria. Luria-Bertani Broth (LB) is a liquid medium used for bacterial growth. Mueller Hinton (MH) and Plate Count Agar (PCA) were used for the antibiotic susceptibility tests, as they are optimized for the used protocol. Lastly, Xylose-lysine-desoxycholate (XLD) - agar was used to distinguish *Escherichia* from *Shigella*, as explained in point 5 of this section.

Table 2. Culture media description

Culture media	Supplier	Composition (g/L)
m-FC agar	VWR	Proteose peptone 5.0; tryptose 10.0; yeast extract 3.0; sodium chloride 5.0; bile salts 1.5; lactose 12.5; methyl blue (formerly aniline blue) 0.1; agar-agar 15.0
Chromocult	Merck (Darmstadt, Germany)	Peptones 3.0; sodium chloride 5.0; sodium dihydrogen phosphate 2.2; di-sodium hydrogen phosphate 2.7; sodium pyruvate 1.0; tryptophan 1.0; agar-agar 10.0; Sorbitol 1.0; Tergitol® 7 0.15; 6-chloro-3 indoxyl-beta-D-galactopyranoside 0.2; isopropyl-beta-D-thiogalactopyranoside 0.1
Luria-Bertani Broth (LB)	Nzytech (Portugal)	Yeast extract 5.0, peptone from casein 5.0, and sodium chloride 10.0
Mueller-Hinton (MH)	Merck (Darmstadt, Germany)	Meat infusion 2.0; casein hydrolysate 17.5; starch 1.5; agar-agar 13.0.
Plate count agar (PCA)	Merck (Darmstadt, Germany)	Casein peptone 5.0, yeast extract 2.5, glucose 1.0, agar 9-18.0

Xylose-lysine-desoxycholate (XLD) agar	Merck (Darmstadt, Germany)	Meat extract 5.0; peptone from meat 5.0; lactose 10.0; sodium thiosulfate 5.4; ammonium iron (III) citrate 1.0; sodium citrate 6.0; sodium deoxycholate 3.0; neutral red 0.02; agar-agar 12.0

2. Sampling and cefotaxime-resistant bacteria isolation

Water samples were collected in September 2018 (in the scope of a previous work) and in September 2019 from Lis river in fifteen sites distributed along the river from the spring to the base level. The sites can be visualized in figure 8 and details are presented in table 3. Water was collected in sterile bottles and kept on ice for transportation. Furthermore, some parameters were measured on site, such as pH, temperature, conductivity and dissolved oxygen, using a portable multi-log environmental meter (WTW, Germany). Additional water quality parameters were also evaluated by collecting 5L samples, which were sent to a credited laboratory for further analysis. The water samples for each of the fifteen sites were filtrated in triplicate through 0.45-µm-pore membranes (Pall Life Sciences) and placed on mFC agar supplemented with 4 µg/mL of cefotaxime (Sigma-Aldrich, USA). The volume of water in each filtration varied between 5 and 50mL. In order to determine the proportion of cefotaxime resistant bacteria, mFC agar without antibiotic was used and inoculated by filtering in triplicate 100µL to 1mL of water. Plates were then incubated at 37 ºC and counts were performed after 24 h. Individual cefotaxime-resistant colonies were purified and stored in 96 well plates with 20% glycerol at -80 ºC.

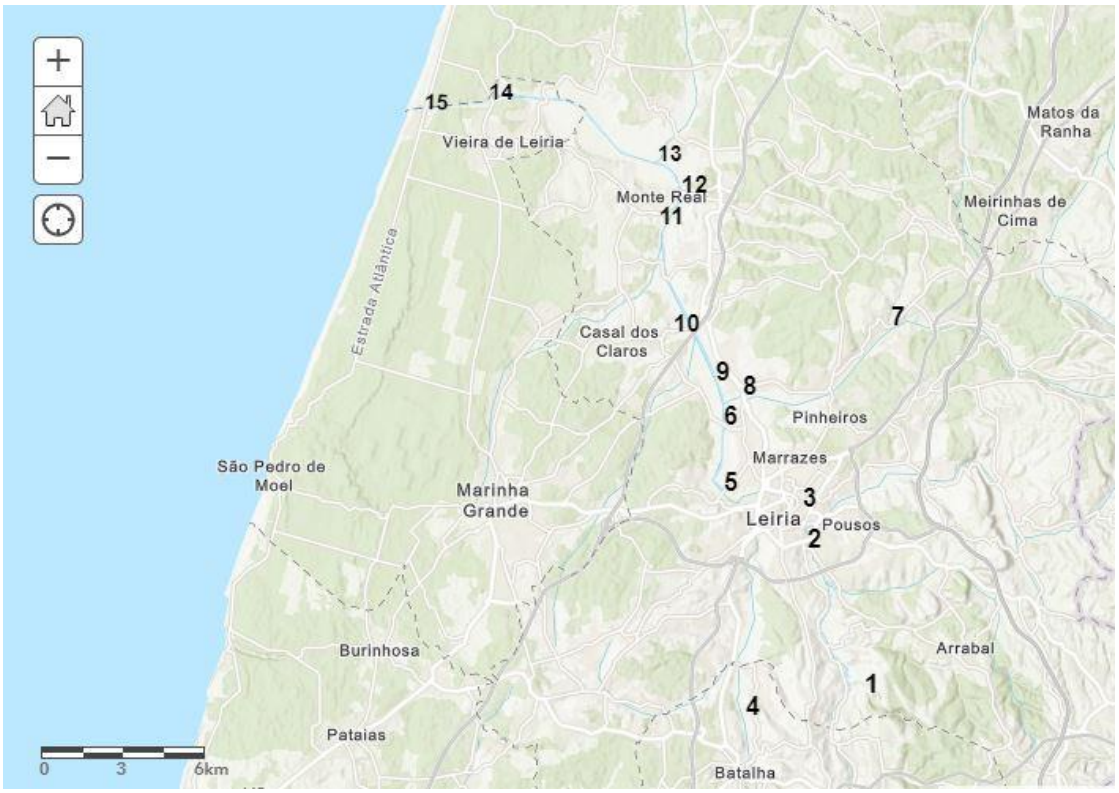


Figure 8. Representation of the sites where the samples were collected along the Lis river (Leiria region). The sites are shown with the numbers 1 (in the spring of the river) to 15 (in the mouth of the river).

Table 3. Coordinates and other information about the sites where samples were collected.

Site	Latitude	Longitude	Observation
1	N 39º41'08	O 8º46'19	Lugar das Fontes (Lis River Spring)
2	N 39º44'	O 8º47'5	Lis River (before the Olhalvas WWTP)
3	N 39º44'24	O 8º47'58	Lis River (after the Olhalvas WWTP)
4	N 39º40'39.7	O 8º49'25.1	Lena river (affluent)
5	N 39º44'49	O 8º49'33.6	Lena river (affluent)
6	N 39°45'09.2	O 8°50'00.2	Lis river
7	N 39º48'27	O 8º45'40.1	Ribeira dos Milagres (affluent)
8	N 39º47'04	O 8º49'29	Ribeira dos Milagres (affluent)
9	N 39º47'21	O 8º50'11.9	Lis river
10	N 39º48'20.5	O 8º51'20.3	Colector de Amor (affluent)
11	N 39º50'28.3	O 8º51'42.9	Colector de Amor (affluent)
12	N 39º51'07.5	O 8º51'08	Lis river
13	N 39º51'43.7	O 8º51'43.0	Lis river
14	N 39º52'59.5	O 8º56'04.8	Lis river
15	N 39º52'49.3	O 8º57'47.2	Lis river (near the Vieira de Leiria beach)

3. 16S rRNA gene sequencing for identification

All isolates (n=147) were retrieved from 96-well plates by placing 2 µL of the glycerol suspension in mFC agar with cefotaxime. For each isolate purity was confirmed and individual colonies were used for amplification of the 16S rRNA gene by PCR. A bacterial cell suspension for each isolate was prepared with 20 µL of dH_2O with an individualized colony. PCR amplification was carried out in a final volume of 25 µL containing 1 µL of cell suspension, 16.25 µL dH_2O, 6.25 µL NZYTaq 2× Green Master Mix (2.5 mM $MgCl_2$; 200 µM dNTPs; 1.25 U DNA polymerase) (NZYTech, Portugal), and 0.75 µL of each primer (forward and reverse) from a stock solution with a concentration of 10 µM. Conditions and primers 27F and 1492R were used as described previously and are presented in table 3 (Lane, 1991), and PCR program is presented in table 4. Negative control was included in the PCR experiment and the resulting products were loaded to an electrophoresis gel at 1.5% agarose, stained with ethidium bromide and visualized using a UV transilluminator. Gel images were acquired with the Image Lab software (Bio-Rad Laboratories, Richmond, CA, USA). Products of the 16S rRNA gene PCR were purified and sent for sequencing (GATC, Konstanz, Germany) and obtained sequences were used for identification by comparing them to the GenBank nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide/) using the BLASTn software (https://blast.ncbi.nlm.nih.gov/Blast.cgi), being previously analyzed using BioEdit (Hall, 1999).

PCR products purification protocol for the 16S rRNA gene:

The PCR products were purified with NZYGelpure (Nzytech, Portugal) following the instructions as described below.

1. 100 µL of Binding Buffer were added to 20 µL of a PCR sample. After mixing, the sample was transferred to a Nzytech Spin Column placed in a 2 mL Receiver Tube and then centrifuged at 12000 x g for 1min.

2. The column was re-inserted in the empty Receiver Tube and 600 µL of Wash Buffer was added, and then centrifuged at 12000 x g for 1min.

3. The flow-through was discarded and the column was placed in the same but empty Receiver Tube, and centrifuged again at 12000 x g for 1 min.

4. The column was placed in to a 1.5 L tube. 25 µL of sterile water was added to the column and centrifuged at 12000 x g for 1 min.

5. The resulting purified DNA was stored at -20ºC.

4. Amplification of *bla*$_{CTX-M}$ gene

All 147 isolates were tested for the presence of the *bla*$_{CTX-M}$ gene, using the same protocol to obtain the template DNA as described above in section 3. Conditions and primers for amplification of the *bla*$_{CTX-M}$ gene were used according to previous studies (Henriques et al., 2006) and can be seen in table 4 and the PCR program is presented in table 5. Positive and negative controls were used in all PCR reactions and the electrophoresis conditions and visualization were the same used in the previous point. Carriers of the *bla*$_{CTX-M}$ gene were then stored at -80ºC with 20% glycerol.

Table 4. Primer description for all PCR experiments

Target	Amplicon size (bp)	Primers sequence (5'-3')	Reference
16S rRNA gene	1467	27F: AGAGTTTGATCCTGGCTCAG	(Lane, 1991)
		1492R: GGYTACCTTGTTAACGACTT	
***bla*$_{CTX-M}$**	538	CTX_F: GTGCAGTACCAGTAAAGTTATGG	(Henriques et al., 2006)
		CTX_R: CGCAATATCATTGGTGGTGCC	
BOX element	Variable	BOX A1R: CTACGGCAAGGCGACGCTGACG	(Versalovic et al., 1991)
ERIC element	Variable	ERIC1: AAGTAAGTGACTGGGGTGAGC	
		ERIC2: ATGTAAGCTCCTGGGGATTCAC	
ISEcp1	Variable	TTCAAAAAGCATAATCAAAGCC	(Eckert et al., 2006)
IS26		CAAAGTTAGCGATGAGGCAG	
Orf477		ACTTCAAAAATTATGCCACC	
IS903		CATCATCCAGCCAGAAAGTT	

Table 5. PCR programs description for amplification of the 16S rRNA and bla$_{CTX-M}$ genes

Temperature (ºC)	16S rRNA	CTX-M	
94	3'	5'	
94	1'	15''	30x
55	1'	30''	
72	2'	45''	
72	10'	7'	

PCR products purification protocol was the same as described in point 3.

5. *Escherichia* and *Shigella* distinction

Since the 16S rRNA gene sequence analysis did not allow to identify *Escherichia* and *Shigella* isolates at the genus level, all isolates that affiliated with one of these genera were cultivated in XLD-agar to confirm identification. The plaques were incubated at 37ºC for 24 hours. XLD agar differentiation relies on the degradation of xylose, lactose and sucrose to acid, which causes phenol red to change its colour to yellow. *Escherichia* have the ability to ferment lactose, and consequently will present a yellow colour. In contrast, *Shigella* species are unable to ferment sugars, which causes the pH of the agar to remain alkaline, and *Shigella* colonies will appear red.

6. Molecular typing of *bla*$_{CTX-M}$ positive *Enterobacteriaceae*

BOX-PCR and ERIC-PCR were used to assess clonality among the isolates. BOX elements are mosaic repetitive elements comprised of different combinations of three subunit sequences, *boxA*, *boxB* and *boxC* (Martin et al., 1992). Likewise, enterobacterial repetitive intergenic consensus (ERIC) sequences have a highly conserved central inverted repeat and are situated in noncoding transcribed regions of the chromosome (Olive & Bean, 1999). Primers used, as well as PCR programs to detect these regions, can be seen in tables 4 and 6. Final volumes for the reactions were 25 µL, being 1 µL of cell suspension, 16.25 µL dH_2O, 6.25 µL NZYTaq 2× Green Master Mix (NZYTech, Portugal), and 2 µL of primer for BOX-PCR (from a solution at 10 µM), and 1 µL of each primer for ERIC-PCR (from a solution at 50 µM). PCR products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide and visualized using a UV transilluminator. Gel images were acquired with the Image Lab software (Bio-Rad Laboratories, Richmond, CA, USA). The typing profiles were analysed with GelCompar II (Applied Maths).

Table 6. PCR programs for BOX and ERIC PCR

BOX		**ERIC**		
Temperature (ºC)	Time	Temperature (ºC)	Time	
95	5’	**94**	7’	
94	1´	**94**	1’	30x
53	1’	**52**	1’	
65	8’	**65**	8’	
65	16’	**65**	16’	

7. Genomic context of the *bla*$_{CTX-M}$ gene

In order to determine the genomic context of the *bla*$_{CTX-M}$ gene, PCR was used with primers specific for sequences known to be associated with the *bla*$_{CTX-M}$ gene, namely IS*Ecp1* and IS*26* for the upstream region of the gene, and orf477 and IS*903* for the downstream region. For the upstream genes, specific primers (seen in table 4) were used along with the CTX_R primer. Logically, for downstream genes, CTX_F primer was used along with the orf477 or IS*903* primer (table 4) depending on the isolate. All isolates were subjected to this analysis, and first we tested the presence of IS*Ecp1* and orf477 (programs in table 7). Then, the samples which did not show presence of these regions were tested for IS*26* and IS*903* (programs in table 6). Positive controls were also used for every reaction which had a final volume of 25 µL, such as described in 3. PCR products were then purified (using the same protocol as in 3) and sent for sequencing, being then assembled using BioEdit. The variant of the gene was found by comparing our results against the CARD (https://card.mcmaster.ca/analyze/blast) as well as Genbank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) databases. Other components associated with the gene were studied using ORF finder (https://www.bioinformatics.org/sms2/orf_find.html) and ISfinder (https://isfinder.biotoul.fr/blast.php).

Table 7. PCR programs used to determine the genomic context of the bla$_{CTX-M}$ genes

Temperature (ºC)	**ISEcp1**	**Orf477**	**IS26**	**IS903**	
94	5’				
94	30’’				30x
50	30’’				
72	2’				
72	7’				

8. Antibiotic susceptibility testing

Susceptibility to antibiotics was tested against 16 antibiotics (Oxoid™) (Table 8) by the disk diffusion method on MH agar according to the procedure established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; https://eucast.org/). Isolates were grown on PCA agar by streaking and incubated overnight at 37 ºC, and then the following protocol was used:

1- Prepare a bacterial suspension in sterile saline solution (0.9% NaCl w/v) with a turbidity equivalent to 0.5 in McFarland scale (3-4 colonies).

2- Use the bacterial suspension to inoculate plates with MH Agar using a swab.

3- After the agar plates dry, apply the disks with antibiotic.

4- Incubate at 37ºC, 18-24 hours.

5- Measure the diameters of the circles of growth inhibition to each antibiotic.

6- Compare the measured diameters with the breakpoints established by EUCAST (v.10.0, 2020) and classify each strain as sensitive, intermediate, or resistant.

The control quality strain used was *E. coli* ATCC25922.

Table 8. List of antibiotics used in the antibiotic susceptibility testing

Antibiotic class or subclass	Antibiotic	Concentration
Penicillin	Amoxicillin (AML)	10 µg
	Amoxicillin/Clavulanic acid (AMC)	30 µg
	Piperacillin (PRL)	30 µg
	Piperacillin/Tazobactam (TZP)	36 µg
	Ticarcillin (TIC)	75 µg
	Ticarcillin/Clavulanic acid (TIM)	85 µg
Cephalosporin	Cefepime (FEP)	30 µg
	Ceftazidime (CAZ)	10 µg
	Cefotaxime (CTX)	5 µg
Monobactam	Aztreonam (ATM)	30 µg
Carbapenem	Imipenem (IPM)	10 µg
Aminoglycosides	Gentamicin (CN)	10 µg
Fluoroquinolones	Ciprofloxacin (CIP)	5 µg
Tetracyclines	Tetracycline (TE)	30 µg
Chloramphenicol	Chloramphenicol (C)	30 µg
Sulfonamides	Trimethoprim/Sulfamethoxazole (SXT)	25 µg

1. Water quality analysis

The water quality data is presented in tables 9 and 10 for all sampled sites along the Lis river. The colours represent water quality classification of each parameter (i.e. blue - Excellent, green - Good, yellow – Reasonable, orange – Poor and red – Very Poor) according to the Portuguese national information system of hydric resources (https://snirh.apambiente.pt/snirh/_dadossintese/qualidadeanuario/boletim/tabela_classes.php; Table S1). The overall classification of each sampling site was attributed according to the classification of the parameter with the worst result.

In 2018 (Table 9) the water quality of all sites was classified as Very Poor, with the exception of site 1 (the spring of the river) which was classified as Poor. A total of 9 sites showed Very Poor results in at least 3 parameters (sites 3, 4, 6, 7, 8, 9, 10, 11 and 14), with site 7 being the worst with 6 parameters evaluated as Very Poor (dissolved oxygen, COD, ammoniacal nitrogen, total nitrogen, nitrates and phosphorous). The parameters dissolved oxygen, total nitrogen and phosphorous were generally above the limits established by the classification scheme mentioned above. Dissolved oxygen levels were classified as Very Poor in all sites except in site 1, the source of the river, in which this parameter was classified as Poor. Total nitrogen levels were also classified as Poor (sites 1 and 3) and Very Poor (the remaining sites except site 2). Phosphorous concentrations were generally above the established limits, being classified as Very Poor in seven sampling sites. Regarding the microbiological parameters, the levels of coliforms, fecal coliforms and enterococci were classified as Reasonable or Good in all sites.

In 2019 (Table 10) water quality was classified as Very Poor in all sampled sites, including the site located near the Spring of the river (site 1). In three sites, three or more parameters were classified as Very Poor (i.e. sites 7, 14 and 15) with site 7 being again the worst case, with seven parameters classified as Very Poor (dissolved oxygen, COD, nitrates, nitrites, total suspended solids, phosphorous and phosphates). Dissolved oxygen concentrations and phosphates concentration were classified as Very Poor in all sites. In terms of microbiological parameters, levels were classified as Good or Reasonable for all sites although levels of total coliforms were generally higher than the ones registered in 2018. Exceptions were sites 4, 8 and 10.

Table 9. Water quality analysis of samples collected in 2018. The data shows the values measured for each site (1 to 15) along the Lis river. Each color represents water quality classification in which: blue - excellent, green - good, yellow – reasonable, orange –poor and red – really poor (https://snirh.apambiente.pt/snirh/_dadossintese/qualidadeanuario/boletim/tabela_classes.php).

Parameters	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Temperature (ºC)	16.0	14.2	14.7	13.3	13.8	14.7	10.8	13.9	14	13.6	14.3	14.4	14.4	15.9	14.9
pH	7.4	8.1	7.9	8.1	8.0	7.9	7.8	7.8	8.2	7.5	7.6	8.0	8.0	7.8	7.8
Conductivity (µS/cm)	0.574	0.524	0.416	0.654	0.654	0.614	0.502	0.254	0.578	0.756	0.544	0.554	0.565	0.592	0.624
Dissolved oxygen (%)	42.3	19.5	19.6	23.1	23.2	17.5	21.1	20.6	21.4	9.7	13.3	19.3	18.7	22.3	24.0
Chemical oxygen demand (COD) (mg/l)	< 5.0	8	30	30	39	24	135	50	10	42	52	18	24	25	28
Copper (mg/l)	< 0.001	0.003	0.006	0.01	0.01	0.007	0.06	0.01	0.002	0.01	0.02	0.005	0.006	0.005	0.005
Ammoniacal Nitrogen (mg/l)	< 0.05	< 0.05	1	0.3	0.3	0.9	22	1	0.2	0.8	0.8	0.4	0.4	0.6	0.5
Total Nitrogen (mg/L)	2.8	1.6	2.4	3.1	3.8	4.3	43	6.1	4.4	13	9.7	5.2	5.5	6	5.1
Biochemical oxygen demand (BOD) (mg/l)	< 3	3	6	3	< 3	4	7	3	< 3	3	5	< 3	< 3	< 3	< 3
Nitrates (mg/l)	6.8	< 1.0	15	12	16	17	68	27	17	52	38	23	23	23	20
Nitrites (mg/l)	< 0.02	< 0.02	1.4	0.7	0.2	0.3	7.3	2.1	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02
Total suspended solids (mg/l)	< 3	12	29	180	43	63	36	120	25	24	34	25	27	26	41
Dissolved Iron (mg/l)	< 0.002	0.01	0.03	0.02	0.03	0.03	0.5	0.1	0.009	0.1	0.2	0.03	0.03	0.05	0.04
Phosphorus (mg/l)	< 0.05	0.06	0.6	0.2	0.3	0.5	1.9	0.7	0.2	1.1	0.5	0.3	0.4	0.6	0.4
Zinc (mg/l)	0.004	0.01	0.03	0.05	0.06	0.04	0.3	0.3	0.008	0.1	0.04	0.02	0.02	0.02	0.02
Fecal Coliforms CFU/100ml	1.9E+02	3.8E+02	7.7E+03	4.0E+03	2.0E+03	6.9E+03	4.0E+03	7.9E+03	4.2E+03	1.9E+03	6.0E+03	3.9E+03	5.9E+03	6.2E+03	4.2E+03
Total Coliforms CFU/100ml	3.9E+03	5.0E+03	8.7E+03	7.1E+03	4.9E+03	9.1E+03	5.5E+03	9.3E+03	8.7E+03	2.5E+03	9.3E+03	7.5E+03	7.9E+03	9.5E+03	8.7E+03
Enterococci CFU/100ml	2.1E+02	7.1E+03	7.1E+03	5.9E+03	7.1E+03	9.1E+03	9.0E+02	8.1E+03	1.5E+03	2.0E+03	3.4E+03	2.2E+03	1.8E+03	1.8E+03	1.7E+03
Overall Water Classification	Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor

Table 10. Water quality analysis data from 2019 The data shows the values measured for each site (1 to 15) along the Lis river. Each color represents a different quality measure in which: blue - excellent, green - good, yellow – reasonable, orange –poor and red – really poor (https://snirh.apambiente.pt/snirh/_dadossintese/qualidadeanuario/boletim/tabela_classes.php)

Parameters	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Temperature (ºC)	18.7	18.1	17.4	17.2	18.1	18.6	19.0	16.5	19.4	19	19.8	19.3	20.0	20.3	19.4
pH	7.6	7.8	7.7	7.4	7.6	7.6	7.3	7.6	7.5	7.6	7.6	7.5	7.6	7.2	7.6
Conductivity (µS/cm)	0.6	0.6	0.5	1	0.9	1.4	0.6	0.5	1.0	0.9	0.9	0.8	0.9	1.0	1.4
Dissolved oxygen (%)	10.5	7.1	6.2	5	6.1	5	5.1	5.8	6.6	4.7	3.8	5.8	6.2	4.2	3.9
Chemical oxygen demand (COD) (mg/l)	< 5.0	< 5.0	8	11	5	8	182	10	31	18	28	70	12	21	< 25
Copper (mg/l)	< 0.001	< 0.001	0.001	< 0.001	0.001	0.001	0.05	0.002	0.001	0.001	0.002	0.002	0.001	0.002	0.001
Biochemical oxygen demand (BOD) (mg/l)	< 3	< 3	< 3	< 3	< 3	< 3	17	< 3	< 3	< 3	6	< 3	< 3	< 3	< 3
Nitrates (mg/l)	12	9	19	7	17	23	96	41	68	35	11	29	40	22	99
Nitrites (mg/l)	<0.1	<0.1	0.2	<0.1	0.4	0.3	1.4	0.2	0.2	<0.1	<0.1	<0.1	0.1	0.5	0.6
Total suspended solids (mg/l)	< 10	< 11	< 11	21	< 11	< 10	503	< 11	< 10	< 13	32	17	< 13	25	< 10
Phosphorus (mg/l)	< 0.05	< 0.05	0.5	0.2	0.3	0.3	3.5	0.5	0.5	0.4	0.3	0.4	0.3	0.6	0.6
Zinc (mg/l)	0.004	0.005	0.01	0.01	0.007	0.004	0.3	0.006	0.01	0.009	0.01	0.01	0.008	0.01	0.01
Fecal coliforms CFU/100ml	4.30E+04	1.30E+04	9.80E+03	2.90E+02	6.00E+04	2.50E+04	3.40E+04	3.20E+02	8.60E+03	1.50E+02	1.20E+02	1.30E+03	1.70E+03	1.10E+03	1.10E+04
Total coliforms CFU/100ml	5.00E+04	3.50E+04	4.30E+04	6.20E+03	9.90E+04	4.00E+04	1.00E+05	6.60E+03	2.10E+04	7.60E+02	3.60E+04	3.40E+04	2.30E+04	2.30E+04	5.20E+04
Enterococci CFU/100ml	5.30E+04	2.30E+03	3.50E+03	4.00E+02	5.30E+03	1.90E+03	1.30E+04	2.50E+02	6.00E+02	2.00E+02	4.00E+01	3.10E+02	1.10E+02	2.30E+03	2.10E+02
Phosphates (mg/l P2O5)	1.3	1.3	2.8	1.9	2	2.9	21.2	3.7	2.7	2.8	2.3	2.4	2.3	2.4	1.9
Overall water classification	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor	Very Poor

2. Abundance of cefotaxime resistant bacteria along the river

In figure 9 are presented the bacterial counts obtained for each site in m-FC agar (figure 9A) and in m-FC agar supplemented with 4 µg/mL of cefotaxime (figure 9B).

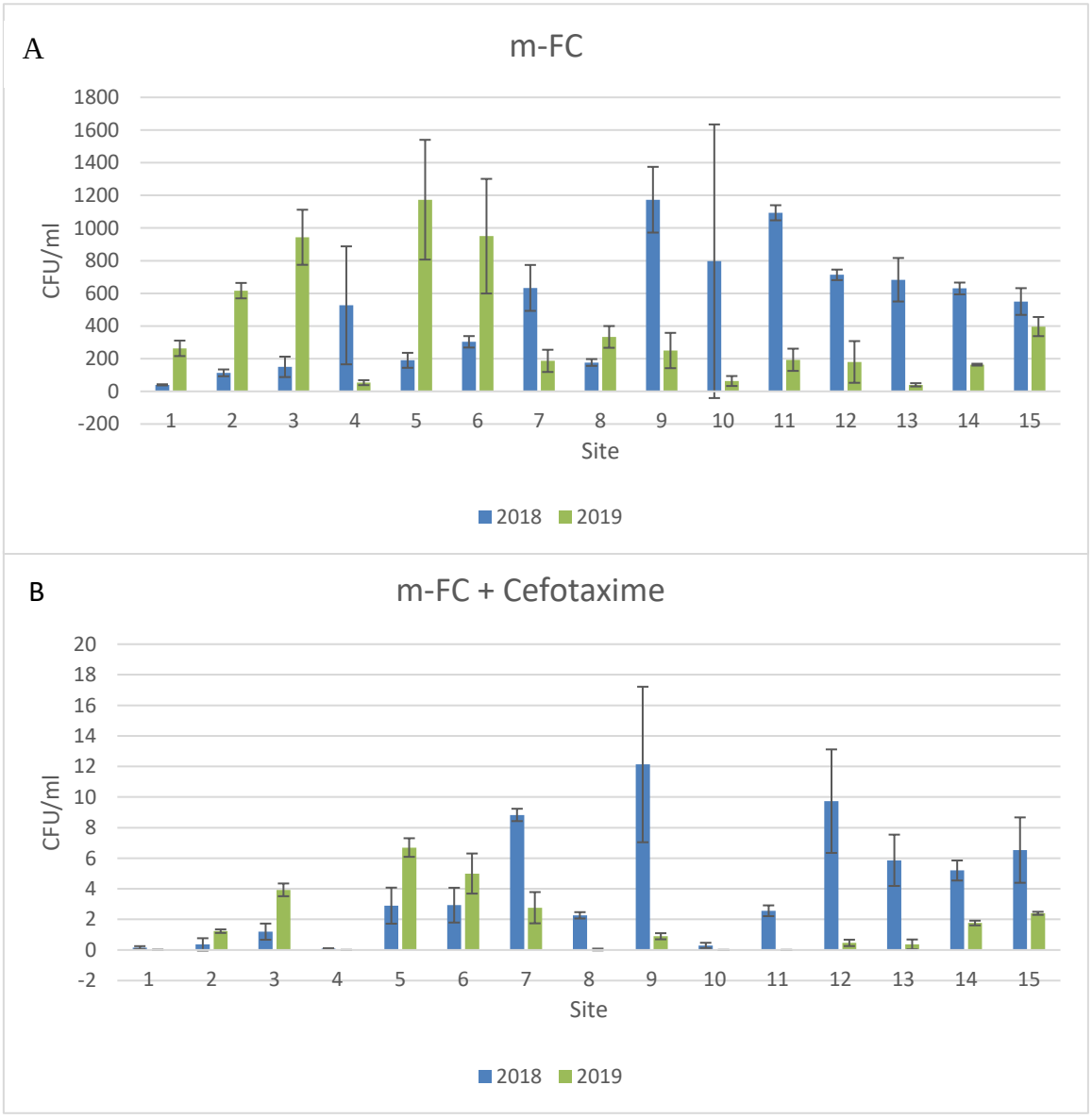


Figure 9. Bacterial counts of CFUs per volume of water sample (mL) in each site (1 to 15). Data from the bacterial counts in m-FC agar are represented in (A) while counts in m-FC agar + cefotaxime are in (B). Each graph shows a comparison between data obtained in 2018 (blue) and in 2019 (green).

Low numbers of CFUs were registered in site 1 (near the spring), both in 2018 and 2019, and in mFC and mFC supplemented with cefotaxime. In 2018, low levels of CFUs in mFC (below 200 CFUs per mL on average) were also registered for sites 2, 3, 5 and 8, while the highest levels (above 1000 CFUs/mL on average) were registered in sites 9 and 11. In 2019, a number above 1000 CFUs/mL on average was registered only for site 5. Regarding the counts of CFUs on mFC supplemented with cefotaxime, those were generally below 8 CFUs/mL, except for sites 7, 9 and 12 in 2019, where the highest values were registered.

In figure 10, we can see the percentage of cefotaxime-resistant bacteria along the river in 2018 and 2019. Data from the 2018 campaign revealed the occurrence of cefotaxime-resistant bacteria in all sites. However, in sites 4 (0.019%), 10 (0.038%) and 11 (0.235%) the lowest prevalences were registered in 2018. In 2019 cefotaxime-resistant bacteria were not detected in those sites. The highest prevalence values in 2018 were registered in sites 5, 7, 8, 12 and 15 with 1.5%, 1.4%, 1.3%, 1.4% and 1.2% respectively. In 2019 the highest values were registered in sites 7 (1.5%), 13 (0.9%) and 14 (1.1%). Values were higher in 2018 in almost all sites, apart from sites 7, 13 and 14. Differences between years was particularly evident in sites 8, with a 1.3% difference, and site 12 with a 1.1% difference.

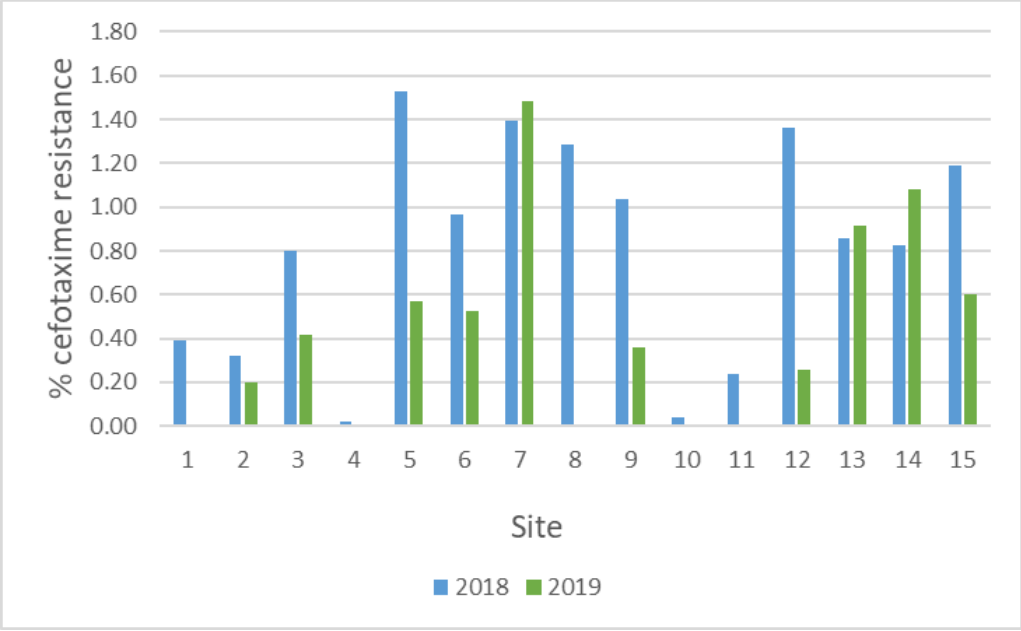


Figure 10. Cefotaxime-resistant bacteria prevalence along the river in all 15 sites, showing the 2018 (blue) and 2019 (green) data

3. 16S rRNA gene-based identification of cefotaxime-resistant bacteria

One hundred and forty-seven cefotaxime-resistant isolates were selected for further analysis. After 16S rRNA gene-based identification, a total of 9 different genera were identified, and 7 of them belonged to the *Enterobacteriaceae* family, namely *Escherichia*, *Klebsiella*, *Citrobacter*, *Enterobacter*, *Shigella* and *Pantoea*. Non-*Enterobacteriaceae* genera were *Acinetobacter*, *Aeromonas* and *Pseudomonas*. Since the 16S rRNA gene sequence is not sufficient to discriminate *Shigella* and *Escherichia* isolates, those that affiliated to these genera were further confirmed by cultivating them in XLD agar. All isolates originated yellow colonies confirming their affiliation to the *Escherichia* genus.

In the 2018 samples all 8 genera were found, whilst in the 2019 campaign *Pantoea* isolates were not detected. Predominant genera varied between years as shown in table 11. In 2018 (91 selected isolates), *Acinetobacter* dominated representing 35% of the total collection, followed by *Escherichia* (32%), *Klebsiella* (12%) and *Pseudomonas* (10%). In 2019 (56 isolates), *Acinetobacter* was represented by only 1 isolate, while *Klebsiella* was the dominant genus (30%), along with *Escherichia* (30%). *Aeromonas* isolates also showed a higher prevalence in 2019, representing 16% of the isolates selected in this year in contrast to 1% in 2018.

Table 11. Genus affiliation of cefotaxime-resistant isolates, collected in 2018 (91 isolates) and 2019 (56 isolates), based on the 16S rRNA gene sequencing.

Genus	Number of isolates collected in 2018 (% of the total)	Number of isolates collected in 2019 (% of the total)
Escherichia*	29 (32)	17 (30)
Klebsiella	11 (12)	17 (30)
Acinetobacter	32 (35)	1 (2)
Citrobacter	3 (3)	5 (9)
Enterobacter	5 (5)	5 (9)
Aeromonas	1 (1)	9 (16)
Pseudomonas	9 (10)	2 (4)
Pantoea	1 (1)	0
Total number of isolates	91	56

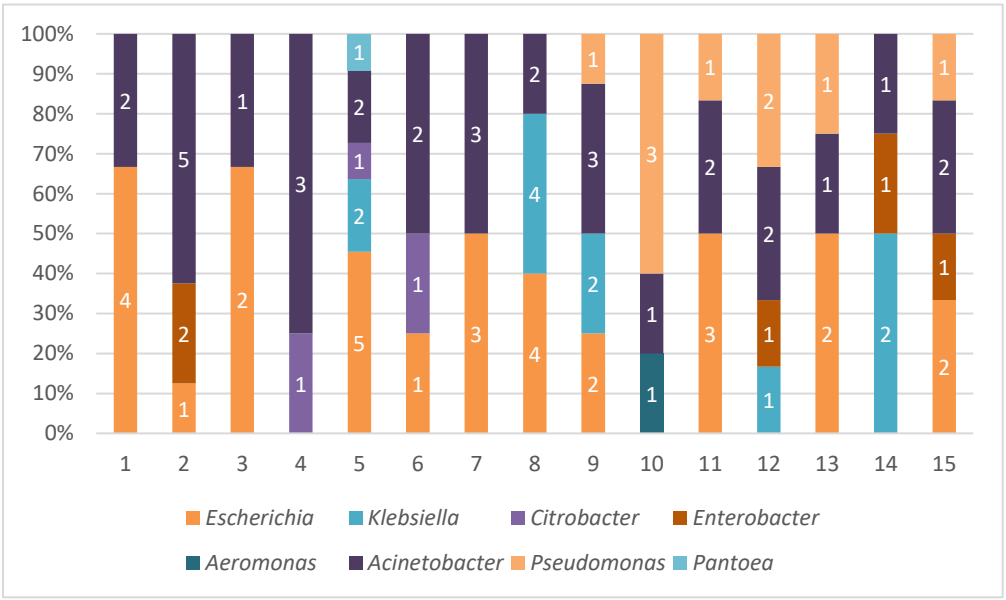
*Based on 16S rRNA gene sequencing and cultivation on XLD agar

When comparing the diversity among all sites (figure 11), *Acinetobacter* isolates were present in all sites in 2018 while the genus was only detected in sites 12 and 15 in 2019. *Escherichia* was detected in 11 sites in 2018 and in 7 sites in 2019, and *Klebsiella* was detected in 5 sites in 2018 and 8 sites in 2019. Site 5 showed the highest diversity with 5 genera

represented, with 4 of them belonging to the *Enterobacteriaceae* family. On the other hand, in sites 3 and 4 isolates from only 2 genera were detected, and from site 4 only one *Enterobacteriaceae* isolate, a *Citrobacter*, was retrieved.

In figure 11 (B), we see the distribution of genera by site in 2019. No isolates were retrieved from sites 4, 10 and 11. *Klebsiella* isolates were the most distributed, appearing in 8 different sites (2, 3, 5, 6, 9, 12, 13 and 14), while *Escherichia* isolates were present in 7 sites (2, 5, 6, 7, 8, 9 and 15). Sites 1, 7 and 8 presented the lowest diversity, contrasting with site 12 with a total of 5 different genera (3 from the *Enterobacteriaceae* family), followed by site 5 with 4 different genera.

A



B

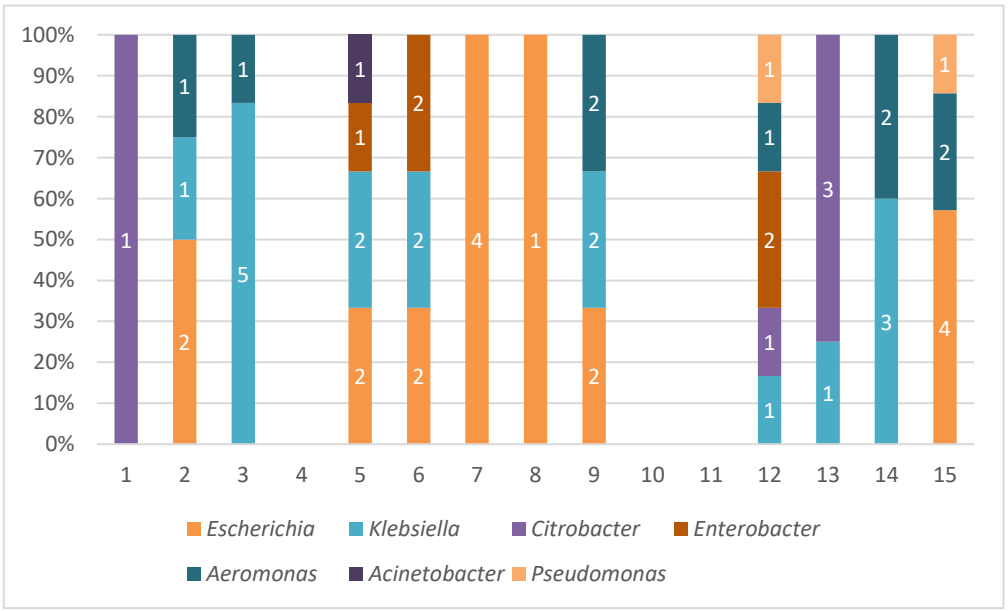


Figure 11. Diversity of isolates retrieved from each site (1 to 15) in 2018 (A) and 2019 (B), with the number of isolates for each genus represented in bars.

4. Prevalence of *bla*$_{CTX-M}$ gene among the cefotaxime-resistant isolates

The *bla*$_{CTX-M}$ gene was inspected by PCR in all the selected isolates. Isolates carrying this gene were obtained from almost all sampled sites along the river in both years of sampling, as seen in figure 12. Nonetheless, from the initial total number of isolates, 68 of them showed the presence of the *bla*$_{CTX-M}$ gene distributed in 6 different *Enterobacteriaceae* genera, namely *Escherichia* (n = 39 *bla*$_{CTX-M}$ positive isolates), *Klebsiella* (n = 24), *Citrobacter* (n = 2) and *Enterobacter* (n = 3). As shown in the graph (figure 12), there was a higher percentage of isolates with the *bla*$_{CTX-M}$ gene in 2019 than in 2018, except for site 1, where *bla*$_{CTX-M}$ was not detected among isolates selected from this site in 2019. The highest prevalence values were determined for sites 7 and 8 with all isolates obtained from these sites in 2019 (n = 6 and 10, respectively) carrying the gene. In 2018 the highest values were obtained for sites 1, 3, 5 and 8.

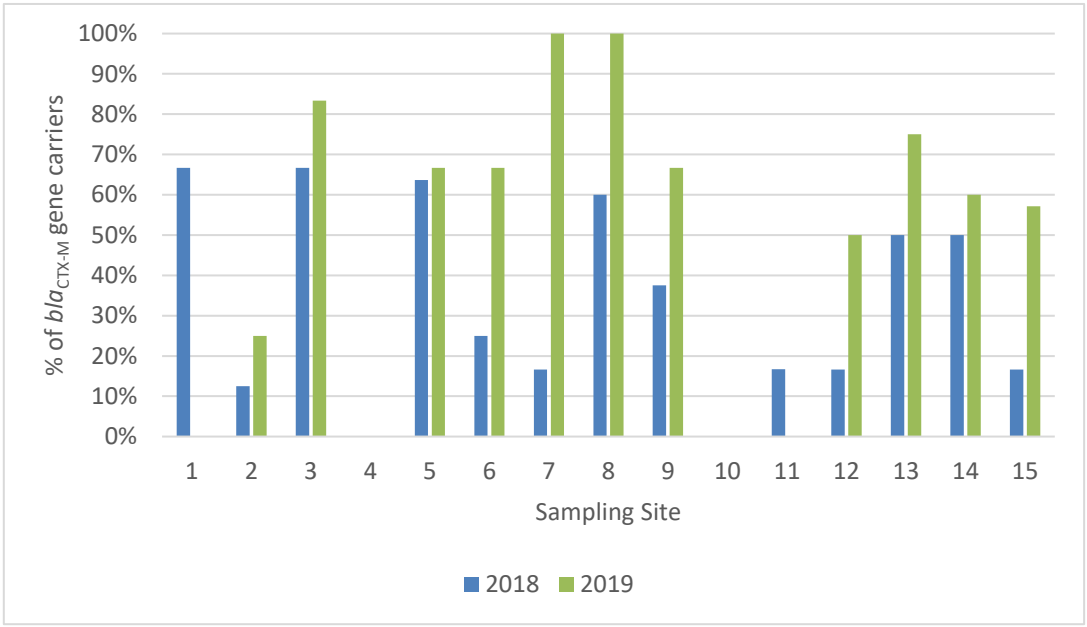


Figure 12. Comparison of the % of *bla*$_{CTX-M}$ gene carriers among cefotaxime-resistant isolates selected in 2018 and 2019 across all sampled sites.

Note that these results are a proportion of carriers, and so the number of isolates for each site influences the outcome. For that reason, in figure 13 are shown the absolute numbers of *bla*$_{CTX-M}$ gene carriers per sampling site in both years. The highest number of *bla*$_{CTX-M}$ carriers was obtained in 2019 (n = 36) when compared to 2018. Among sites, the highest number of *bla*$_{CTX-M}$ carriers was obtained from site 5 (7 isolates in 2018 and 4 isolates in 2019), followed by site 3 (2 isolates in 2018 and 5 isolates in 2019), site 8 (6 isolates in 2018 and 1 isolate in 2019) and site 9 (3 isolates in 2018 and 4 isolates in 2019).

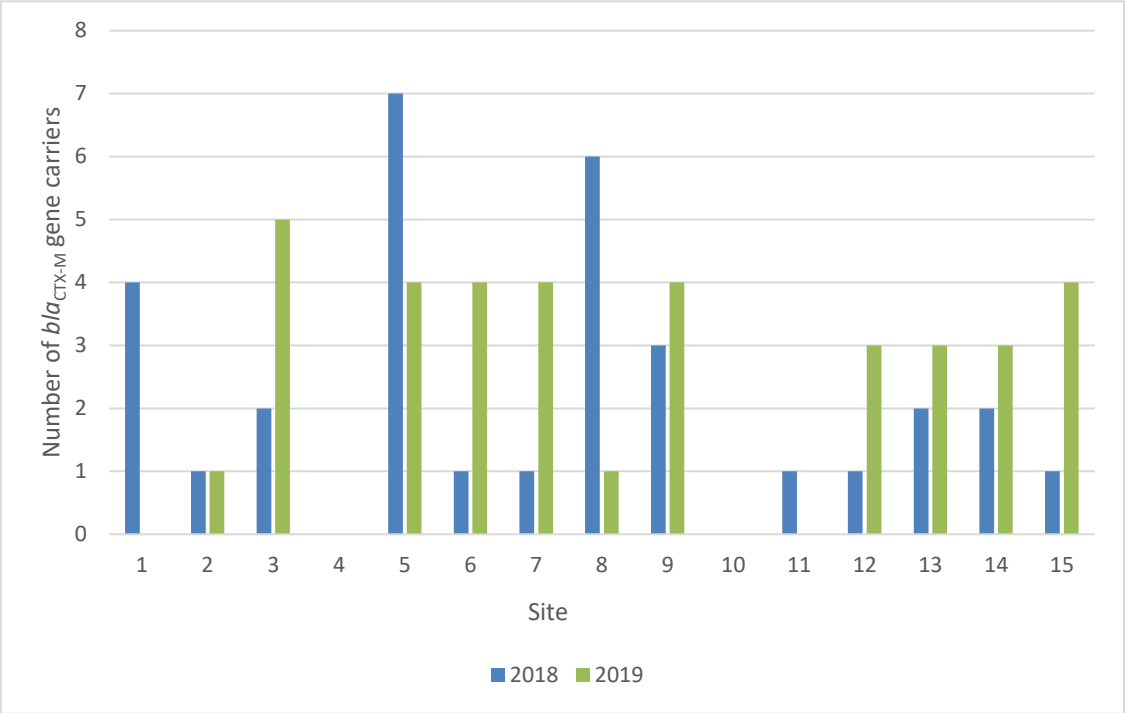


Figure 13. Number of isolates with the *bla*$_{CTX-M}$ gene across all sites, collected in 2018 and 2019.

The distribution of *bla*$_{CTX-M}$ carriers among the identified genera (figure 14) showed a clear dominance of *Escherichia* with 57% of the total number of isolates carrying this gene (n=32), followed by *Klebsiella* with 35%. *Enterobacter* with 5% and *Citrobacter* with 3% complete the identified genera with the *bla*$_{CTX-M}$ gene.

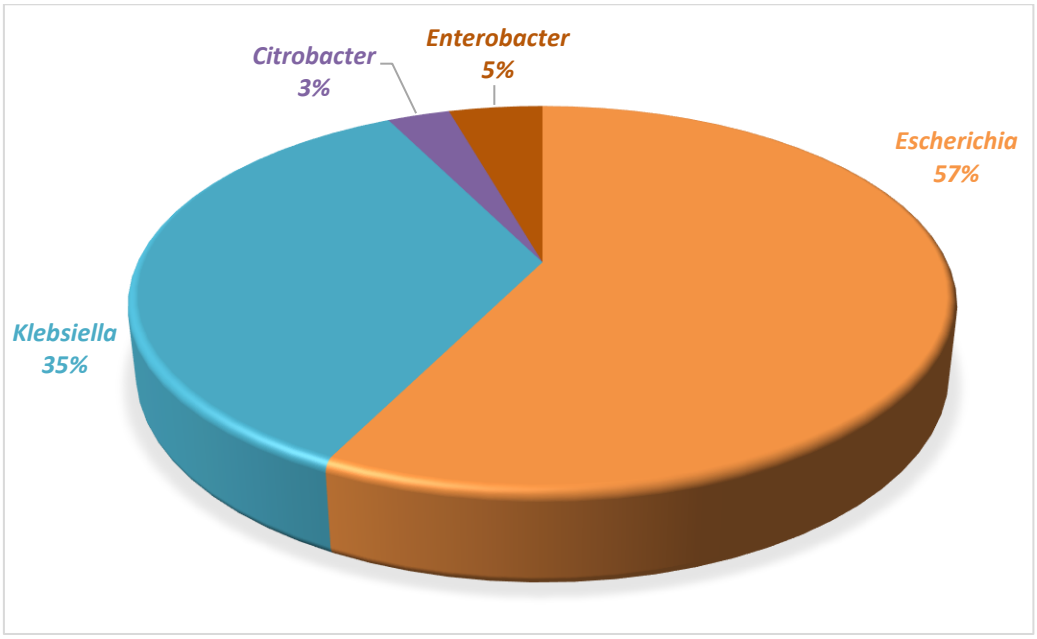


Figure 14. Genera affiliation of *bla*$_{CTX-M}$ gene carriers

5. Characterization of *bla*$_{CTX-M}$ carriers along the river

The clonality of all isolates was evaluated with BOX and ERIC PCR analysis. If isolates from the same site and the same year presented identical BOX and ERIC profiles, only one was selected for further analysis.

The BOX and ERIC PCR results can be observed in figure 15 and figure 16. Isolates marked in red were excluded from further analysis as they present highly similar BOX and ERIC profiles when compared to other isolates from the same site in the same year. However, some isolates presented identical profiles but were present in different sites and were selected for further analysis. These include *Escherichia* 2.1, and 9.1, from 2018 (figure 15), that present identical BOX and ERIC profiles, to those obtained for *Escherichia* 7.1, and 15.3 (isolated in 2019). Another example concerns *Escherichia* isolate 5.1 from 2019 that had identical BOX and ERIC profiles to those obtained for *Escherichia* 6.5 and 9.4, both from 2019 as well. Likewise, *Klebsiella* isolates 6.3 and 5.3 isolated in 2018 and 2019, respectively, share identical profiles.

As a result, 54 isolates, in which some might be clones, were selected for further analysis from the initial 68 gene carriers, and the majority were *Escherichia* (32 isolates), followed by *Klebsiella* (18 isolates), and 3 *Enterobacter* isolates and only 1 *Citrobacter*.

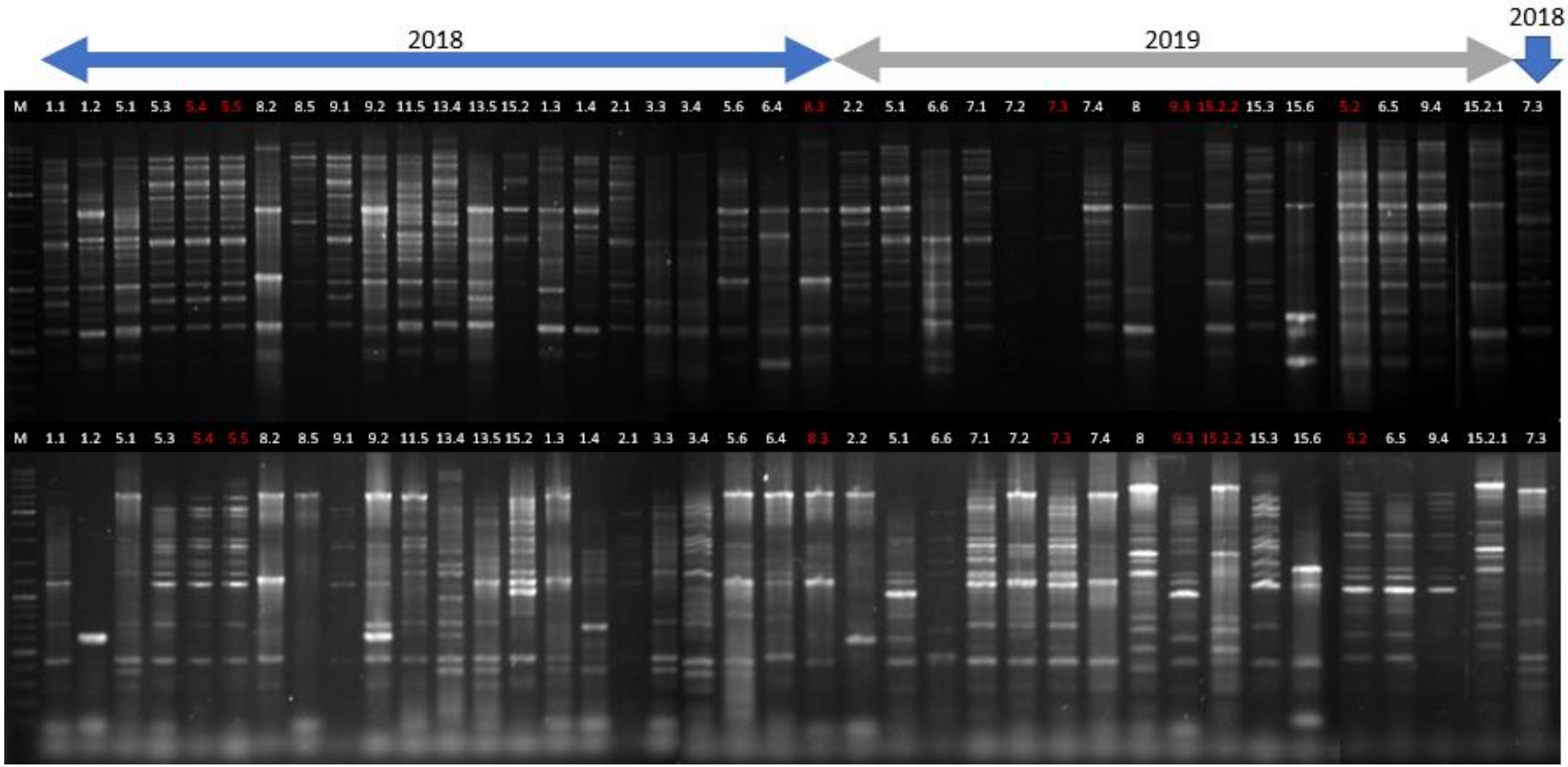


Figure 15. BOX and ERIC profiles for all *Escherichia* isolates. The first number indicates the site and the second is isolate specific. In some cases, a third number represents a duplicate. The results on top are from BOX PCR and the ones on bottom correspond to ERIC profiles. Isolates in red represent the excluded ones.

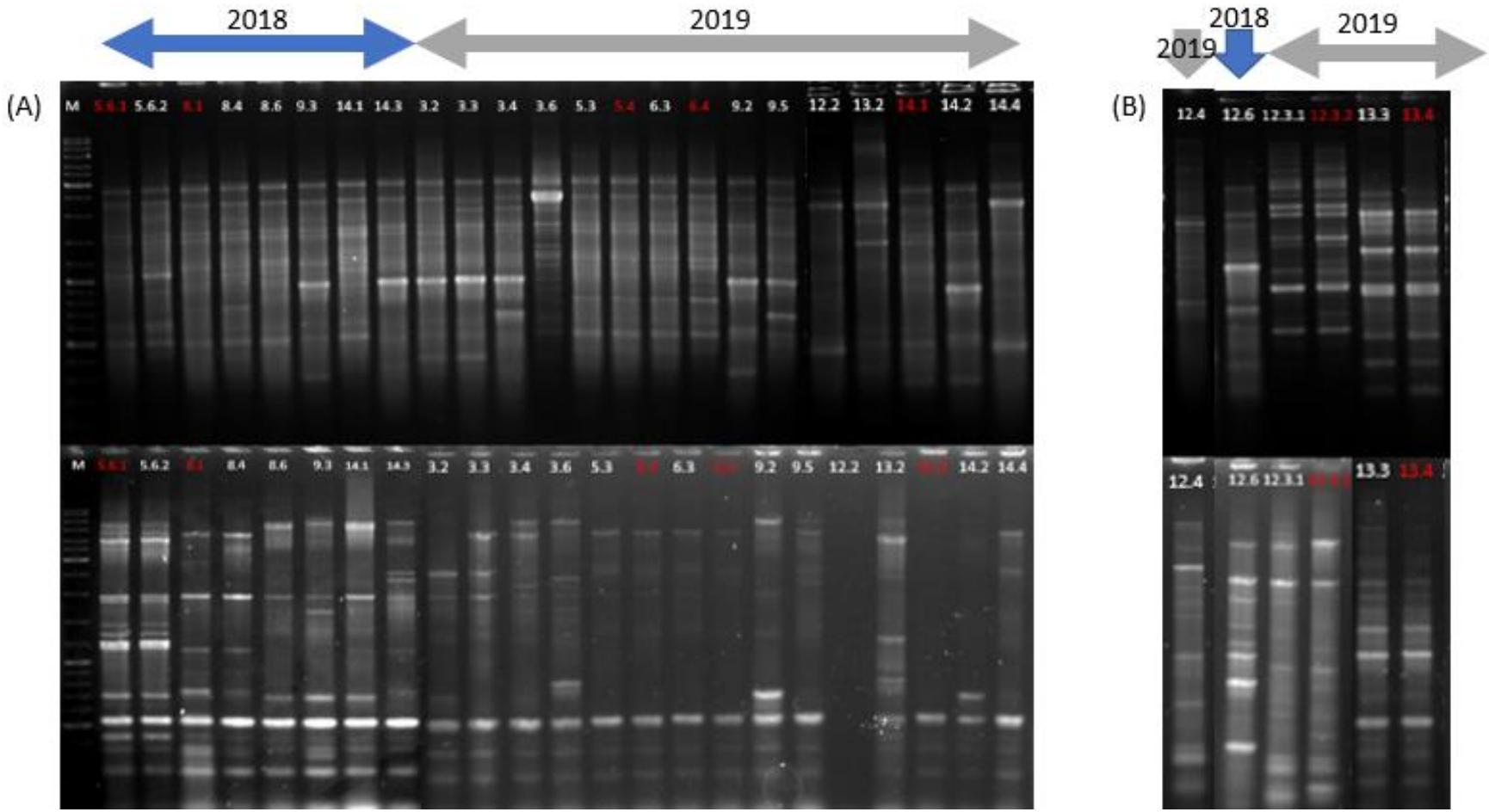


Figure 16. BOX and ERIC profiles for *Klebsiella* (A), *Enterobacter* and *Citrobacter* (B) isolates. The first number indicates the site and the second is isolate specific. In some cases, a third number represents a duplicate. The results on top are from BOX PCR and the ones on bottom correspond to ERIC profiles. Isolates in red represent the excluded ones. *Citrobacter* isolates are addressed as 13.3 and 13.4, the remaining isolates in (B) are Enterobacter.

6. Antibiotic susceptibility testing

Antibiotic resistance phenotypes were evaluated for all 54 isolates. Results can be seen for *Escherichia* isolates in table 12, for *Klebsiella* isolates in table 13 and for *Enterobacter* and *Citrobacter* in table 14. Isolates were classified as resistant, intermediate and susceptible, as mentioned before in the materials and methods section. For analysis purposes, isolates with intermediate results were addressed as resistant.

Table 12. Antibiotic susceptibility profiles for the *Escherichia* isolates, as well as the site from which they were retrieved and the gene variant corresponding to each isolate (isolates from 2018 are in green and from 2019 in yellow). Darker color classifies an isolate as resistant, medium as intermediate and lighter color as susceptible. Antibiotics: AML- amoxicillin, AMC – amoxicillin/clavulanic acid, PRL – piperacillin, TZP - piperacillin/tazobactam, TIC – ticarcillin, TIM – ticarcillin/clavulanic acid, CTX – cefotaxime, CAZ - ceftazidime, FEP - cefepime, ATM - aztreonam, CIP - ciprofloxacin, CN - gentamicin, TET - tetracycline, C - chloramphenicol, SXT - trimethoprim/sulfamethoxazole

Site	Strain	Profile[#]	*bla*$_{CTX-M}$	AML	AMC	FEP	PRL	TZP	ATM	CAZ	CTX	TIC	TIM	IPM	CN	CIP	TE	C	SXT
1	E1	1.1	UN*																
1	E2	1.2	*bla*$_{CTX-M-1}$																
5	E3	5.1	*bla*$_{CTX-M-32}$																
5	E4	5.3	*bla*$_{CTX-M-65}$																
7	E5	7.3	*bla*$_{CTX-M-1}$																
8	E6	8.2	*bla*$_{CTX-M-1}$																
8	E7	8.5	*bla*$_{CTX-M-15}$																
9	E8	9.1	*bla*$_{CTX-M-65}$																
9	E9	9.2	UN*																
11	E10	11.5	*bla*$_{CTX-M-14}$																
13	E11	13.4	UN*																
13	E12	13.5	*bla*$_{CTX-M-15}$																
15	E13	15.2	*bla*$_{CTX-M-15}$																
1	E14	1.3	UN*																
1	E15	1.4	*bla*$_{CTX-M-55}$																
2	E16	2.1	*bla*$_{CTX-M-65}$																
3	E17	3.3	*bla*$_{CTX-M-15}$																
3	E18	3.4	*bla*$_{CTX-M-15}$																
5	E19	5.6	*bla*$_{CTX-M-32}$																
6	E20	6.4	*bla*$_{CTX-M-15}$																
2	E21	2.2	*bla*$_{CTX-M-15}$																
5	E22	5.1	*bla*$_{CTX-M-27}$																
6	E23	6.5	*bla*$_{CTX-M-27}$																
6	E24	6.6	*bla*$_{CTX-M-55}$																

7	E25	7.1	UN*															
7	E26	7.2	*bla*$_{CTX-M-32}$															
7	E27	7.4	*bla*$_{CTX-M-15}$															
8	E28	8	*bla*$_{CTX-M-15}$															
9	E29	9.4	*bla*$_{CTX-M-27}$															
15	E30	15.2.1	*bla*$_{CTX-M-15}$															
15	E31	15.3	*bla*$_{CTX-M-65}$															
15	E32	15.6	*bla*$_{CTX-M-1}$															

[#]According to Figure 15 and 16; isolates with identical BOX and ERIC profiles are highlighted with the same color.

*Unidentified.

Table 13. Antibiotic susceptibility profiles for the *Klebsiella* isolates as well as the site from which they were retrieved and the gene variant corresponding to each isolate (isolates from 2018 are in green and from 2019 in yellow). Darker color classifies an isolate as resistant, medium as intermediate and lighter color as susceptible. Antibiotics: AML- amoxicillin, AMC – amoxicillin/clavulanic acid, PRL – piperacillin, TZP - piperacillin/tazobactam, TIC – ticarcillin, TIM – ticarcillin/clavulanic acid, CTX – cefotaxime, CAZ - ceftazidime, FEP - cefepime, ATM - aztreonam, CIP - ciprofloxacin, CN - gentamicin, TE - tetracycline, C - chloramphenicol, SXT - trimethoprim/sulfamethoxazole

Site	Strain	Profile[#]	*bla*$_{CTX-M}$	AML	AMC	FEP	PRL	TZP	ATM	CAZ	CTX	TIC	TIM	IPM	CN	CIP	TE	C	SXT
5	K1	5.6.2	*bla*$_{CTX-M-15}$																
8	K2	8.4	UN*																
8	K3	8.6	*bla*$_{CTX-M-15}$																
9	K4	9.3	*bla*$_{CTX-M-15}$																
14	K5	14.1	*bla*$_{CTX-M-15}$																
14	K6	14.3	*bla*$_{CTX-M-15}$																
3	K7	3.2	*bla*$_{CTX-M-15}$																
3	K8	3.3	*bla*$_{CTX-M-15}$																
3	K9	3.4	UN*																
3	K10	3.6	*bla*$_{CTX-M-15}$																
5	K11	5.3	*bla*$_{CTX-M-15}$																
6	K12	6.3	UN*																

9	K13	9.2	UN*																
9	K14	9.5	UN*																
12	K15	12.2	*bla*$_{\text{CTX-M-15}}$																
13	K16	13.2	UN*																
14	K17	14.2	UN*																
14	K18	14.4	*bla*$_{\text{CTX-M-15}}$																

[#]According to Figure 15 and 16; isolates with identical BOX and ERIC profiles are highlighted with the same color.
*Unidentified.

Table 14. Antibiotic susceptibility profiles for the *Enterobacter* (C1-3) and *Citrobacter* (C4) isolates as well as the site from which they were retrieved and the gene variant corresponding to each isolate (isolates from 2018 are in green and from 2019 in yellow). Darker color classifies an isolate as resistant, medium as intermediate and lighter color as susceptible. Antibiotics: AML- amoxicillin, AMC – amoxicillin/clavulanic acid, PRL – piperacillin, TZP - piperacillin/tazobactam, TIC – ticarcillin, TIM – ticarcillin/clavulanic acid, CTX – cefotaxime, CAZ - ceftazidime, FEP - cefepime, ATM - aztreonam, CIP - ciprofloxacin, CN - gentamicin, TE - tetracycline, C - chloramphenicol, SXT - trimethoprim/sulfamethoxazole.

Site	Strain	Profile[#]	*bla*$_{\text{CTX-M}}$	AML	AMC	FEP	PRL	TZP	ATM	CAZ	CTX	TIC	TIM	IPM	CN	CIP	TE	C	SXT
12	C1	12.6	UN*																
12	C2	12.3.1	*bla*$_{\text{CTX-M-3}}$																
12	C3	12.4	UN*																
13	C4	13.3	*bla*$_{\text{CTX-M-32}}$																

[#]According to Figure 15 and 16; isolates with identical BOX and ERIC profiles are highlighted with the same color.
*Unidentified.

Overall results showed that isolates presented resistance levels below 60% only to four out of sixteen antibiotics, namely piperacillin/tazobactam (39%), imipenem (4%), gentamicin (31%) and chloramphenicol (26%). The highest resistance rates were observed for beta-lactams, namely towards cefepime, piperacillin, aztreonam and ticarcillin with 98% each, and 100% of the isolates were resistant to amoxicillin and, as expected, to cefotaxime. Additionally, high resistance levels were observed to ticarcillin/clavulanic acid (87%). followed by ciprofloxacin (80%), amoxicillin/clavulanic acid (78%), ceftazidime (76%), trimethoprim/sulfamethoxazole (72%) and tetracycline with 63%. In figure 17, we can see in more detail the percentage of resistant isolates to each antibiotic by genus.

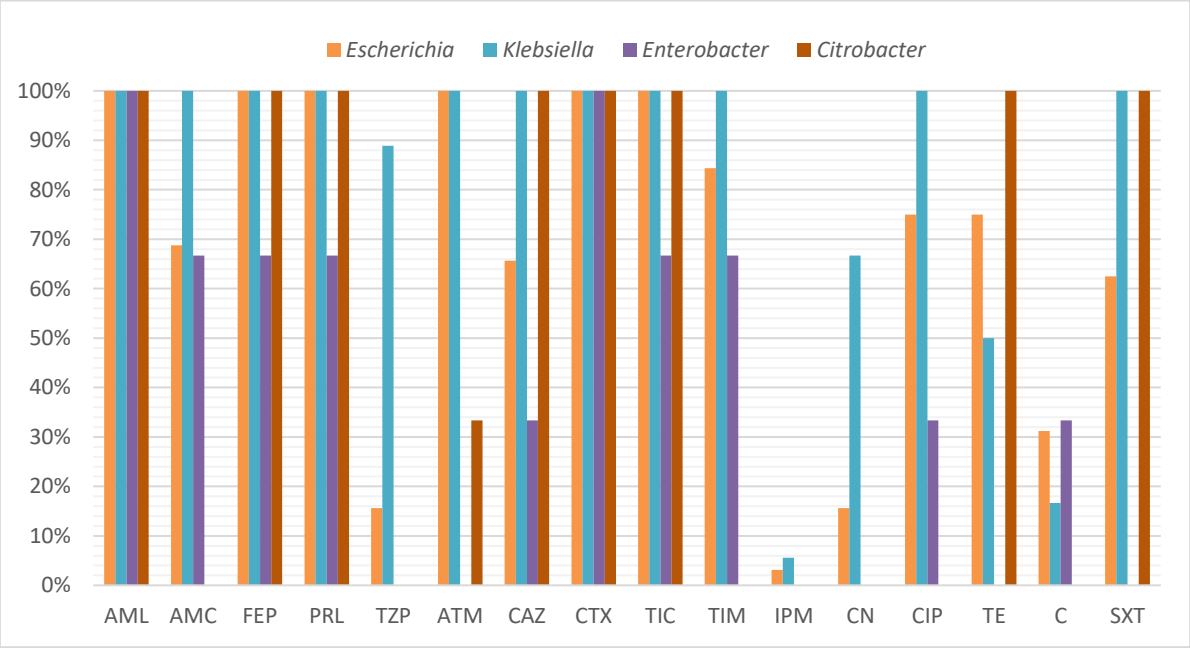


Figure 17. Percentage of resistant isolates in each of the four genera for each antibiotic. Each color represents a genus as presented in the right site of the graphic.

All *Escherichia* isolates were resistant to all penicillins tested, except to the combinations of amoxicillin/clavulanic acid (22 susceptible isolates), piperacillin/tazobactam (5 susceptible isolates) and ticarcillin/clavulanic acid (27 susceptible isolates). *Escherichia* isolates were also resistant to the cephalosporins tested, except to ceftazidime (21 susceptible isolates). The lowest rates of resistance were observed to imipenem (only 1 resistant), gentamicin (5 resistant isolates) and to chloramphenicol (10 resistant isolates).

Klebsiella isolates were resistant to all antibiotics tested, with the exception of imipenem (1 resistant isolate), gentamicin (12 resistant isolates), tetracycline (9 resistant isolates), chloramphenicol (3 resistant isolates) and piperacillin/tazobactam (2 resistant isolates). The isolate

K15 was only susceptible to Imipenem and was the only isolate resistant to all classes of antibiotics tested.

Moreover, *Enterobacter* isolates (C1, C2 and C3) show more susceptibility to the tested antibiotics. The 3 isolates were all resistant to amoxicillin and cefotaxime. C1 and C3 were resistant to 8 out of the 16 antibiotics tested, while C2 was only resistant to 4. Finally, the *Citrobacter* isolate (C4) was resistant to 9 antibiotics in total.

Multiresistance is considered when a bacteria is resistant to antibiotics included in three or more classes (Magiorakos et al., 2011). Keeping this in mind, a total of 79.6% (corresponding to 43 isolates) showed multiresistance phenotypes, and all of them were resistant to at least one β-lactam. All *Klebsiella* isolates were multiresistant, as well as the *Citrobacter* isolate. On the other hand, none of the *Enterobacter* isolates showed multiresistance, with C1 being only resistant to β-lactams, C2 to β-lactams and chloramphenicol, and C3 to β-lactams and ciprofloxacin (fluoroquinolone). Finally, among the *Escherichia* isolates, 24 showed a multiresistance phenotype, with isolates E2, E9 and E25 only revealing resistance to β-lactams, E11 and E15 to β-lactams and tetracycline, and E17, E18 and E20 were resistant to β-lactams and ciprofloxacin.

7. *bla*$_{\text{CTX-M}}$ gene diversity

A total of seven different variants of the *bla*$_{\text{CTX-M}}$ gene were found, as seen in figure 18. The *bla*$_{\text{CTX-M-15}}$ was the most prevalent, being present in 52.5% of all isolates. Among the other variants, *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-32}}$ and *bla*$_{\text{CTX-M-65}}$ were present in 10% of the isolates. *bla*$_{\text{CTX-M-3 and}}$ *bla*$_{\text{CTX-M-14}}$ showed the lowest prevalence, each being present in only 1 isolate. Note that these results only concern 40 of the 54 total isolates, as it was not possible to identify the *bla*$_{\text{CTX-M}}$ variant for the remaining 14 isolates.

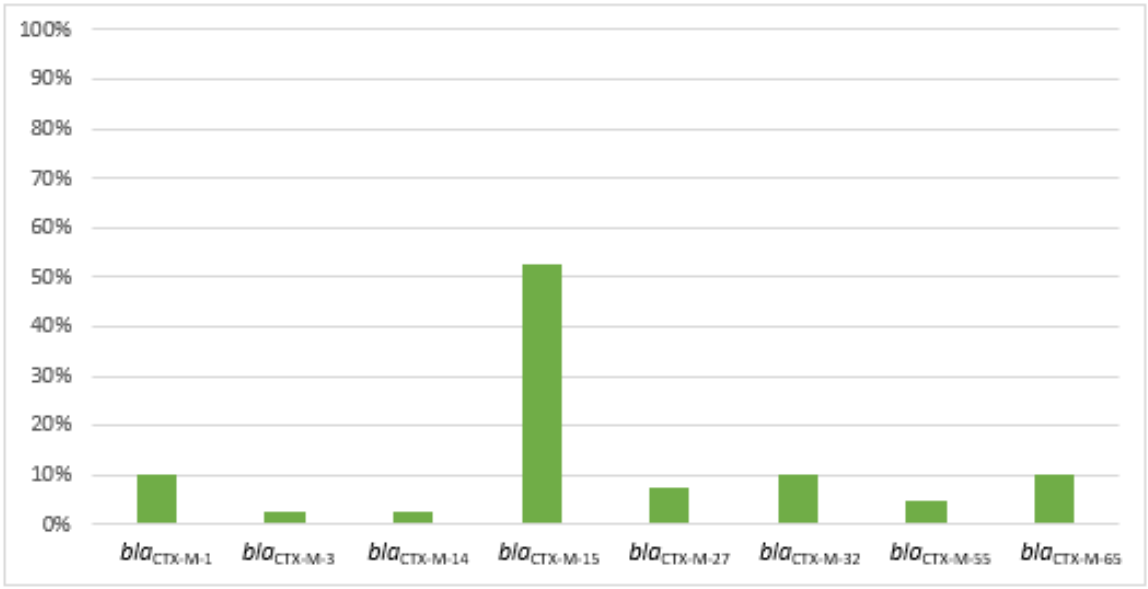


Figure 18. Proportion of all variants of the bla$_{CTX-M}$ gene found in 40/54 isolates

Table 15 presents the distribution of *bla*$_{CTX-M}$ variants among genera with *Escherichia* showing isolates with all variants found, except *bla*$_{CTX-M-1}$, prevailing *bla*$_{CTX-M-15}$ with 10 out of 32 isolates, and the less frequent being *bla*$_{CTX-M-14}$ with only one *Escherichia* isolate showing this variant. *bla*$_{CTX-M-3}$was present in only one *Enterobacter* isolate. *bla*$_{CTX-M-15}$ was present in all *Klebsiella* isolates. The *Citrobacter* carried a *bla*$_{CTX-M-32}$.

Table 15. Number of isolates by variant of the gene from each bacterial group.

Variant	*Escherichia*	*Klebsiella*	*Enterobacter*	*Citrobacter*	Total
***bla*$_{CTX-M-1}$**	4				4
***bla*$_{CTX-M-3}$**			1		1
***bla*$_{CTX-M-14}$**	1				1
***bla*$_{CTX-M-15}$**	10	11			21
***bla*$_{CTX-M-27}$**	3				3
***bla*$_{CTX-M-32}$**	3			1	4
***bla*$_{CTX-M-55}$**	2				2
***bla*$_{CTX-M-65}$**	4				4
Unknown	5	7	2		14
Total	32	18	3	1	54

8. Genomic environment of the *bla*$_{\text{CTX-M}}$ gene

The genomic environment of the different *bla*$_{\text{CTX-M}}$ gene variants is represented in figure 19. All of the variants found share the presence of the insertion sequence IS*Ecp1* in the upstream region of the gene, with variable distances to the gene, mainly due to the presence of different conserved regions. The distance between IS*Ecp1* and the start codon of *bla*$_{\text{CTX-M}}$ genes was as previously described (Eckert et al., 2006; Lartigue et al., 2004; Tacão et al., 2012), varying from 32 bp to 127 bp. *bla*$_{CTX\text{-}M\text{-}15}$ was associated with 3 different environments. In figure 11 (A) and (H), *bla*$_{CTX\text{-}M\text{-}1}$ and *bla*$_{CTX\text{-}M\text{-}32}$ genes show two conserved regions with 32 bp and 48 bp and orf477 in the downstream region of the gene. Figure 19 (B) and (E) represent the environment of the *bla*$_{CTX\text{-}M\text{-}3}$ gene and a *bla*$_{CTX\text{-}M\text{-}15}$ environment variation, with a conserved region of 79 bp and one with 48 bp between the IS and the gene, with orf477 in the downstream region. The flanking regions of the *bla*$_{CTX\text{-}M\text{-}14}$, *bla*$_{CTX\text{-}M\text{-}27}$ and *bla*$_{CTX\text{-}M\text{-}65}$ are represented in figure 19 (C), (G) and (J), respectively, with only one conserved region of 42 bp between the IS and the gene, and with another insertion sequence (IS*903*) in the downstream region. Figure 19 (I) shows the bla$_{CTX\text{-}M\text{-}55}$ variant genetic context, similar to the *bla*$_{CTX\text{-}M\text{-}15}$ in (D), both surrounded by IS*Ecp1* and IS*903*, with a conserved region of 42 bp between the IS*Ecp1* and the gene. Finally, in figure 19 (F) is presented another variation of *bla*$_{CTX\text{-}M\text{-}15}$ environment, identical to that in (D), but it shows an additional insertion sequence IS*26* prior to the IS*Ecp1*.

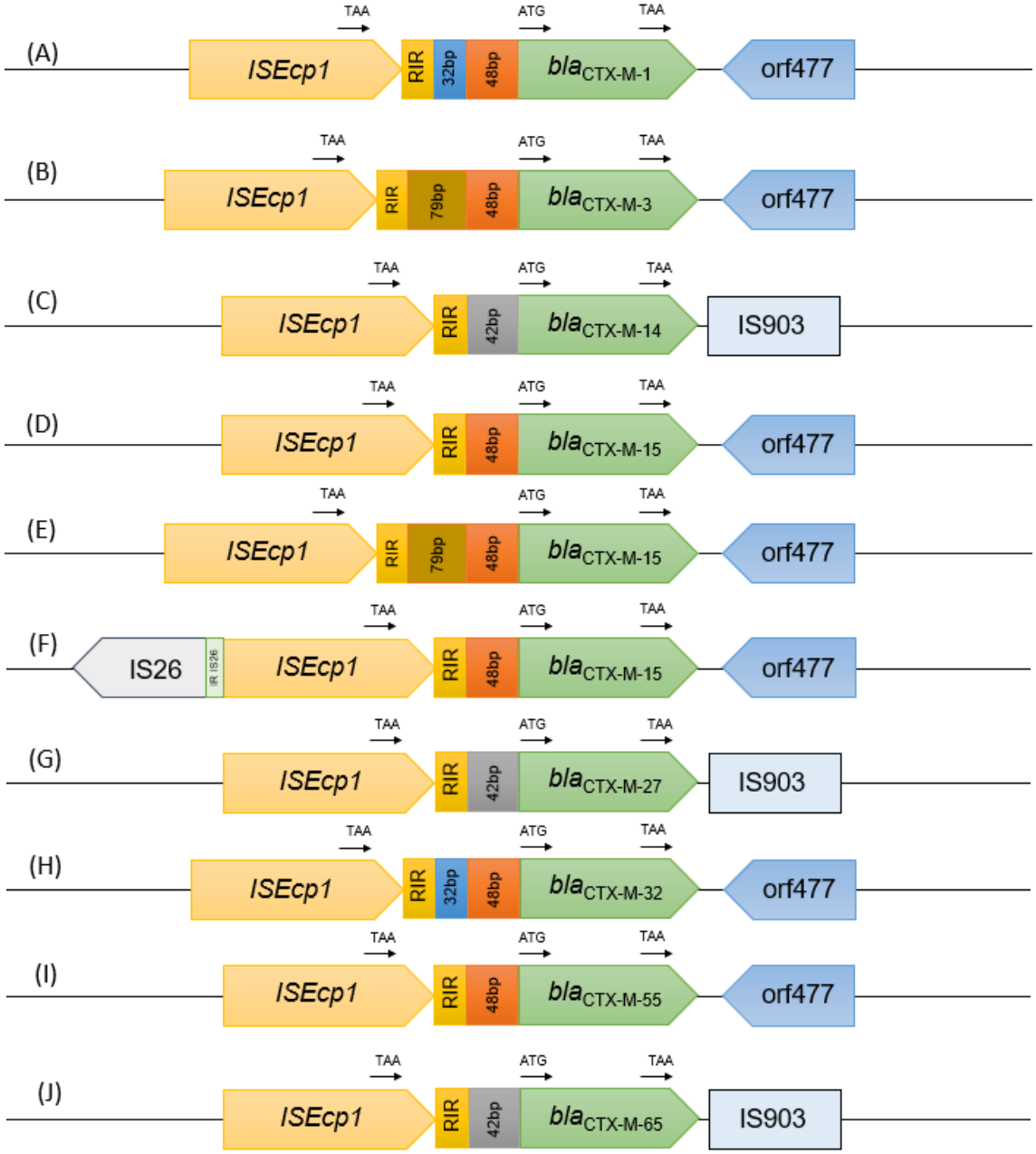


Figure 19. Genomic environments for the variants of the *bla*$_{\text{CTX-M}}$ gene found in the isolates. 10 different environments were observed, and they are represented from (A) to (J).

Discussion

Rivers are considered reservoirs of multiresistant bacteria, since they receive water from all kinds of sources, like WWTPs, industrial effluents, agricultural activities, hospital sewage or animal production effluents (Lupo et al., 2012). All of these examples of contamination have been reported in the Lis river (Fonseca et al., 2014; Paíga et al., 2016; Teixeira et al., 2020; Vieira et al., 2012, 2013; Vieira et al., 2009), and so it is extremely important to understand how the antibiotic resistance problem currently sits in this ecosystem and how it can evolve in the future.

When analyzing the water quality data, some numbers stand out for negative reasons. Total nitrogen levels were concerning, particularly in 2018, all along the river. These values might correlate with the agricultural activities in the river margins as well as the animal production activities along the river. Also, a mention to site 7, that shows the worst overall numbers in both years. In addition to several parameters classified as Very Poor in this site, also the concentrations of metals (i.e. Cu and Zn) were in general higher than in other sampling sites. These compounds are used as supplements for animal food or as pesticides in agriculture (Gräber et al., 2005). Higher metal concentrations may contribute to an antibiotic resistance selection, since genes conferring resistance to metals and antibiotics are sometimes together in the same mobile genetic elements (Mcintosh et al., 2008). There are even cases where efflux mechanisms confer resistance to both antibiotics and metals, known as cross-resistance (Aendekerk et al., 2002; Nishino et al., 2007). Previous studies also revealed that the prevalence of antibiotic resistance is higher in sites polluted by metals (Baker-Austin et al., 2006; Henriques et al., 2016; Ji et al., 2012). This site is located near several piggeries which might be sources of contamination, as well as the agricultural activities in the river margins. Swine slurry is a mixture of pig feces and urine with wastewater and sometimes precipitation that contains mainly suspended solids, nitrogen, phosphorous and potassium (Girard et al., 2009). For site 7, nitrogen levels in 2018 were by far the highest (no data from 2019) and for phosphorous too, while suspended soils even though it had reasonable levels in 2018, in 2019 it was the highest value and classified the site as with very poor quality. Phosphorous levels were high across the river, mainly in sites 3, and then from 6 onwards. As mentioned earlier, phosphorous is an important product of swine slurry (Girard et al., 2009), and is also used as a fertilizer for agricultural purposes. As such, the high values of these components in the river are likely to be a product of these activities. Furthermore, site 3 shows some concerning values in both years, particularly when looking at the phosphorous values, that can be related to the fact that the sampling site is downstream a WWTP combined with being near the hospital, as phosphorus is known to be present in hospital wastewater (Pirsaheb et al., 2015). Previous studies in the Lis river (Vieira et al., 2012, 2013) and Lena river (Fonseca et al., 2014) reported poor water quality in the river, converging with the results from this study.

Even though the m-FC agar is a selective and differential medium and optimized for the growth of *Enterobacteriaceae*, other groups can grow as well. In the 2018 campaign, the criteria for counting bacteria was less selective, considering colonies with distinct colours and morphologies to analyse how these characteristics are associated to each genera, which led to a higher proportion of non-*Enterobacteriaceae* isolates selection in that campaign, in contrast to 2019. In fact, in 2019, more strict criteria were applied in order to select only bacteria belonging to the *Enterobacteriaceae* family. This explains, at least in part, why the number of cefotaxime-resistant bacteria was usually higher in 2018. These methodological differences may also explain the dominance of *Acinetobacter* in 2018, while in 2019 only 1 isolate was retrieved. However, none of the non-*Enterobacteriaceae* isolates showed the presence of the $bla_{\text{CTX-M}}$ gene, but previous studies detected the gene in *Acinetobacter* (Potron et al., 2011; Shakil & Khan, 2010) and *Aeromonas* (Girlich et al., 2011; Maravić et al., 2013; Ye et al., 2010), and they found as well in Portuguese rivers 3 isolates of *Aeromonas hydrophila* producing $bla_{\text{CTX-M-3}}$ (Tacão et al., 2012).

As for the abundance of *Enterobacteriaceae* resistant to cefotaxime along the river, site 7 had the highest value among all sites (figure 2), which is probably due to its location being close to a high number of piggeries, who are a well-known source of untreated effluents that reach the river (Paíga et al., 2016; Vieira et al., 2013). Site 1 represents the spring of the Lis river, showing a small number of isolates resistant to cefotaxime which is consistent with lower levels of pollution at this site. Looking with more attention to the sites that belong to the main course of the Lis river (1, 2, 3, 6, 9, 12, 13, 14 and 15) it is noticeable that the percentage of cefotaxime-resistant *Enterobacteriaceae* has a tendency to increase along the river course. Quick note as well to the sites 10 and 11, which are located in the Amor affluent, and from where no resistant bacteria were isolated, which can be related to lower anthropogenic pressure in that area when compared to other sites.

When comparing the percentage of isolates carrying the $bla_{\text{CTX-M}}$ gene in both years of sampling, it is noticeable that higher values were registered in 2019. Even though these values may have been affected by the different criteria applied in bacteria isolation, the water quality data suggests higher levels of pollution in 2019, and so it is plausible a rise in the prevalence of $bla_{\text{CTX-M}}$ gene carriers. All isolates from sites 7 and 8 carried the $bla_{\text{CTX-M}}$ gene in 2019. Both sites are part of the Ribeira dos Milagres affluent, which is the part of the Lis river most affected by pollution (Vieira et al., 2012). In fact, the presence of the $bla_{\text{CTX-M}}$ gene has been associated with high levels of anthropogenic influence (Tacão et al., 2012). The $bla_{\text{CTX-M}}$ gene appears in sites 1 and 11 in 2018, in contrast to 2019, in which none of the isolates collected from this site revealed the presence of this gene, maybe due to the fact that that these sites are more isolated than the rest.

Escherichia and *Klebsiella*, represent together 93% of all isolates. These two genera include clinically relevant pathogens, and in this case maybe particularly critical due to their ESBL-producing feature and multiresistance. So, their frequent occurrence in Lis river is alarming, as these waters can easily reach the population. Risks to human health arise from the fact that the water from Lis river is frequently used for irrigation, fishing and leisure activities, facilitating the contact of humans with resistant bacteria. Previous studies confirmed the transfer of antibiotic-resistant bacteria from irrigation water to vegetables that are consumed raw (Araújo et al., 2017). Also, previous studies have confirmed the transfer of cefotaxime-resistant bacteria from water to humans during leisure activities (Leonard et al., 2018). Infections caused by resistant strains from these groups of bacteria are growing every year across all continents (Bevan et al., 2017). They are often found in clinical environments (i.e. hospitals) and it is becoming harder to respond to these infections, as there is an urgent need for new antibiotics for these groups according to the WHO (WHO, 2017).

When selecting clonal isolates based on BOX and ERIC profiles, we noticed that even though the majority of the isolates had different profiles in at least one experiment, some of them revealed the same profiles suggesting clonality. This happened in the same site for the same year (the excluded isolates, red colored in figures 7 and 8) and that was the criteria for selection. However, some of the isolates showing identical profiles were collected in different sites and even in different years. This is really interesting, as clonal isolates from the same year in multiple sites may indicate that these strains have the ability to survive in this environment for some period of time, enabling them to travel along the river. If their resistance gene is located in a plasmid, their persistence may promote gene transfer to other bacteria. Furthermore, some isolates showed similar profiles in both years, suggesting that there may be a continuous contamination source, which complies with the rest of the work. However, further studies are needed to confirm these hypothesis.

bla$_{CTX\text{-}M}$ genes are well known for being associated to other antibiotic resistance genes. This derives from the fact that *bla*$_{CTX\text{-}M}$ genes are often located in conjugative plasmids that harbor resistance genes to fluoroquinolones, aminoglycosides and sulfonamides (Gniadkowski, 2001; Tacão et al., 2014). Keeping this in mind it is not surprising that a total of 79.6% were classified as multiresistant. Regarding all isolates, it is noticeable that there are low values of resistance to imipenem (4%). Despite the low value, and the fact that the isolates showed intermediate resistance, it's still very concerning, as carbapenems are used as last resource antibiotics to treat *Enterobacteriaceae* infections (ECDC, 2017), and a recent study shows that the Lis river does in fact have carbapenem-resistant *Enterobacteriaceae* (Teixeira et al., 2020). The other low resistance values were detected for piperacillin with tazobactam (39%), but because tazobactam is a β-lactamase inhibitor, this result was expected. In contrast, both combinations with clavulanic acid (amoxicillin and ticarcillin) showed concerningly high numbers, as clavulanic acid is a β-lactamase inhibitor as well, which shows that these bacteria probably developed defenses against this agent. Low resistance rates to gentamycin can be explained by the fact that gentamycin is not as used as

other antibiotics, as well as the later introduction of this antibiotic in clinic and veterinary medicine (Van Hoek et al., 2011). Chloramphenicol resistance is mainly associated with chloramphenicol acetyltransferases (CATs) (Van Hoek et al., 2011; Wright, 2005), and its low percentage resistance rates may be justified by the lack of these enzymes in the isolates. Also, tetracycline and trimethoprim/sulfamethoxazole are antibiotics commonly used in animal production, and so their high resistance rates, 63% and 72% are not surprising. Furthermore, sulfamethoxazole is one of the principal antibiotics detected in the Lis river water (Paíga et al., 2016). In a general way, *Klebsiella* isolates show the higher resistance rates amongst all isolates, followed by *Escherichia* isolates, and finally *Citrobacter* and *Enterobacter* isolates, which presented lower resistance profiles. This is concerning, since *Klebsiella spp.* and *Escherichia spp.* are the main *Enterobacteriaceae* cause of infections, and *Escherichia* isolates in the river are certainly driven from anthropogenic influences.

The CTX-M enzymes are divided in six main groups (D'Andrea et al., 2013) and are produced from $bla_{CTX\text{-}M}$ genes. In this work, group 1 is represented (CTX-M-1, CTX-M-3, CTX-M-15, CTX-M-32 and CTX-M-55), as well as group 9 (CTX-M-14, CTX-M-27 and CTX-M-65). $bla_{CTX\text{-}M\text{-}15}$ stands out as the most prevalent gene variant among all isolates, with 21 showing the gene that codes this enzyme (52.5% when observing only the 39 tested isolates). The others are evenly distributed, except for $bla_{CTX\text{-}M\text{-}3}$ and $bla_{CTX\text{-}M\text{-}14}$, that show the lowest rate among all. It is also noticeable that only $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}32}$ were detected in different genera, notably $bla_{CTX\text{-}M\text{-}15}$ in *Escherichia* and *Klebsiella, and* $bla_{CTX\text{-}M\text{-}32}$ in *Escherichia* and *Citrobacter*. All group 9 variants show the same genetic environment, starting with IS*Ecp1*, followed by a conserved region of 42 bp and IS*903* in the downstream region of the gene, as seen before in this group (Eckert et al., 2006; Lartigue et al., 2004). The $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}32}$ genetic environment is the same, and the only difference between these variants is a nucleotide substitution within the gene. $bla_{CTX\text{-}M\text{-}15}$ has been identified for the first time in India back in 1999 and since then has become the most prevalent $bla_{CTX\text{-}M}$ gene variant worldwide, in both hospitals and the environment (Karim et al., 2006; Kittinger et al., 2016). It is also the main variant found in Portuguese aquatic systems (Bevan et al., 2017). For that reason, it would be expected that $bla_{CTX\text{-}M\text{-}15}$ would prevail in detriment to other variants. It is important to notice as well that all *Klebsiella* isolates have the exact same variant, first identified in Portugal in a *Klebsiella* isolate (Conceição et al., 2005), probably due to a lower diversity of *Klebsiella* in the river when comparing to *Escherichia* isolates. However, a more in-depth study of these isolates would be necessary. Also, the isolate K15 has IS*Ecp1* disrupted by another insertion sequence, IS26, which might influence as well. As mentioned before, there are few studies regarding the presence of $bla_{CTX\text{-}M}$ in aquatic environments, and even fewer in Portugal. Even so, most variants detected in this work from both groups were previously found in rivers in Portugal (Tacão et al., 2012), although this might be the first report showing the presence of $bla_{CTX\text{-}M\text{-}65}$ in a Portuguese river. Furthermore, to our knowledge, there are only two reports of $bla_{CTX\text{-}M\text{-}27}$ in Portugal, one in a H30 subclone of B2-ST131 *E.coli* retrieved from a Portuguese healthy human (Rodrigues et al., 2016) and another from a *E.coli* isolated in a WWTP in northern Portugal (Silva et al., 2018).

As expected, clonal isolates in different sites and/or year, in general, carried the same *bla*$_{CTX\text{-}M}$ gene.

Final regards and future perspectives

As expected, the data from this work indicate high levels of pollution in the Lis river. As reported in other studies, it suggests that the piggeries represent the main source of pollution, but wastewaters from agricultural activities, as well as hospitals and WWTPs should also be considered. The sites that show more impacts due to pollution also show the higher rates of $bla_{\text{CTX-M}}$ prevalence, which indicates these gene as an important antibiotic pollution marker. The vast majority of $bla_{\text{CTX-M}}$ gene carriers are from clinically relevant bacteria (*Escherichia* and *Klebsiella*) and since a lot of them reveal multiresistance phenotypes is a concerning factor to natural environments as well as public health. Also, this study reports for the first time the presence of $bla_{CTX\text{-}M\text{-}65}$ in Portugal.

Concerning future approaches, it would be interesting to know which other genes are linked with the $bla_{\text{CTX-M}}$ gene, as well as the ability for these bacteria to transfer the gene in between them and others. More studies in this river would also be of interest, as it would be important to make sure of the impacts of piggeries, as well as the evolution of the antibiotic resistant phenotype in the bacteria present in the river and their possible negative impact in human health.

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Appendix

Table S1: Classification of surface water courses according to their characteristics for multiple purposes, according to the Portuguese national information system of hydric resources. The colours represent water quality classification of each parameter (i.e. blue - Excellent, green - Good, yellow – Reasonable, orange – Poor and red – Very Poor)

\\ Classificação dos cursos de água superficiais de acordo com as suas características de qualidade para usos múltiplos.

PARÂMETRO:	UNIDADES:	MÉTODO DE CÁLCULO		A Excelente		B Boa		C Razoável		D Má		E Muito má
		PERCENTIL	FREQUÊNCIA	MIN	MAX	MIN	MAX	MIN	MAX	MIN	MAX	-
Arsénio	mg/l As	85	3	-	0.01	-	0.05	-	-	-	0.1	>0.1
Azoto Kjeldahl	mg/l N	85	4	-	0.5	-	1	-	2	-	3	>3
Azoto amoniacal	mg/l NH4	85	8	-	0.5	-	1.5	-	2.5	-	4	>4
Carência bioquímica de oxigénio	mg/l O2	85	8	-	3	-	5	-	8	-	20	>20
Carência química de oxigénio	mg/l O2	85	8	-	10	-	20	-	40	-	80	>80
Chumbo	mg/l Pb	85	3	-	0.05	-	-	-	0.1	-	0.1	>0.1
Cianetos	mg/l CN	85	3	-	0.05	-	-	-	0.08	-	0.08	>0.08
Cobre	mg/l Cu	85	3	-	0.05	-	0.2	-	0.5	-	1	>1
Coliformes fecais	/100 ml	85	8	-	20	-	2000	-	20000	-	>20000	
Coliformes totais	/100 ml	85	8	-	50	-	5000	-	50000	-	>50000	
Condutividade	µS/cm, 20ºC	85	8	-	750	-	1000	-	1500	-	3000	>3000
Crómio	mg/l Cr	85	3	-	0.05	-	-	-	0.08	-	0.08	>0.08
Cádmio	mg/l Cd	85	3	-	0.001	-	0.005	-	0.005	-	>0.005	
Estreptococos fecais	/100 ml	85	4	-	20	-	2000	-	20000	-	>20000	
Fenois	mg/l C6H5OH	85	4	-	0.001	-	0.005	-	0.01	-	0.1	>0.1
Ferro	mg/l Fe	85	3	-	0.5	-	1	-	1.5	-	2	>2
Fosfatos P2O5	mg/l P2O5	85	8	-	0.4	-	0.54	-	0.94	-	1	>1
Fósforo P	mg/l P	85	8	-	0.2	-	0.25	-	0.4	-	0.5	>0.5
Manganês	mg/l Mn	85	3	-	0.1	-	0.25	-	0.5	-	1	>1
Mercúrio	mg/l Hg	85	3	-	0.0005	-	-	-	0.001	-	0.001	>0.001
Nitratos	mg/l NO3	85	8	-	5	-	25	-	50	-	80	>80
Oxidabilidade	mg/l	85	8	-	3	-	5	-	10	-	25	>25
Oxigénio dissolvido (sat)	% saturação de O2	85	8	90	-	70	-	50	-	30	-	<30
Selénio	mg/l Se	85	3	-	0.01	-	-	-	0.05	-	0.05	>0.05
Substâncias tensioactivas	mg/l, sulfato de lauril e sódio	85	4	-	0.2	-	-	-	0.5	-	0.5	>0.5
Sólidos suspensos totais	mg/l	75	8	-	25	-	30	-	40	-	80	>80
Zinco	mg/l Zn	85	3	-	0.3	-	1	-	3	-	5	>5
pH	Escala Sorensen	85	8	6.5	8.5	5.5	9	5	10	4.5	11	>11

- O pH, sendo um parâmetro muito dependente de características geomorfológicas, pode apresentar valores fora deste intervalo, sem contudo significar alterações de qualidade devidas à poluição.
- Alteração de frequência ao Azoto Kjeldahl desde 2006.
- Nas classificações referentes ao Norte em 2007 não foi respeitada a frequênica mínima de amostragem.