Universidade de Aveiro Departamento de Biologia
Ano 2014/2015

Ana Margarida Barros do Coito

Bacterial communities and antibiotic resistance genes in hospital effluents and municipal wastewaters of Aveiro

Comunidades bacterianas e genes de resistência a antibióticos em efluentes hospitalares e em águas residuais de Aveiro

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Sónia Alexandra Leite Velho Mendo Barroso, Professora auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro e da Doutora Isabel da Silva Henriques, Investigadora Auxiliar do iBiMED e Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho aos meus queridos pais António do Coito e Ana Barros, assim como, ao meu irmão Paulo, pelo apoio constante e motivação.

o júri

Presidente

Prof. Doutora Maria Ângela Sousa Dias Alves Cunha
Professora auxiliar do Departamento de Biologia da Universidade de Aveiro

Doutora Marta Cristina Oliveira Martins Tacão
Investigadora em Pós-doutoramento do Departamento de Biologia da Universidade de Aveiro

Prof. Doutora Sónia Alexandra Leite Velho Mendo Barroso
Professora auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro

agradecimentos

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palavras-chave

Resistência a antibióticos; genes de resistência a antibióticos; comunidades microbianas; ambiente hospitalar; águas residuais.

resumo

O ambiente hospitalar é caracterizado por uma grande diversidade de diferentes espécies bacterianas, as quais podem tornar-se resistentes a antibacterianos. Apesar da natureza específica de efluentes hospitalares, estes são considerados com a mesma carga poluente e como constituintes das águas residuais urbanas, sendo descarregados para redes de esgotos urbana, recolhidos e cotratados com os efluentes municipais na ETAR.

O objetivo do estudo consistiu em avaliar a estrutura das comunidades microbianas e determinar a ocorrência e abundância de genes relacionados com resistência a antibióticos (*bla*$_{CTX-M}$, *bla*$_{VIM}$, *bla*$_{TEM}$, *bla*$_{SHV}$ e *intI*1) de efluentes hospitalares (internamento e laboratório de microbiologia) e efluentes urbanos (pré e pós-tratamento na ETAR de Cacia). Procedeu-se com a análise por DGGE e por PCR quantitativa.

As amostras foram colhidas em duas estações diferentes, revelando as diferenças na estrutura da comunidade bacteriana. As comunidades dos efluentes das hospitalizações e dos efluentes de pré e pós-tratamento ficaram agrupadas no mesmo *cluster*, enquanto as comunidades predominantes nos efluentes do laboratório de microbiologia localizaram-se num outro cluster. Os cinco genes selecionados estavam presentes em todos os efluentes. Os efluentes hospitalares tiveram a maior abundância total dos genes do que os efluentes urbanos. De todos os genes, *bla*$_{VIM}$ teve uma abundância notável devido a sua alta abundância de águas residuais nas hospitalizações e enfermarias, seguido por *IntI*1. *bla*$_{CTX-M}$, *bla*$_{TEM}$ e *bla*$_{SHV}$ demonstraram abundancias mais baixas. No que diz respeito ao tratamento biológico aplicado na ETAR de Cacia, uma vez que a abundância de genes foi menor após o tratamento, é previsível que o tratamento possa ser moderadamente eficaz.

Os nossos resultados mostram que os ambientes hospitalares tendem a ser um ambiente complexos devido à elevada diversidade microbiana, às pressões seletivas criadas pelo uso de antimicrobianos e pelos eventos de transferência lateral de genes. A rede de esgotos urbanos e da ETAR de Cacia representam reservatórios de resíduos de antibióticos, determinantes de resistência a antibióticos e bactérias resistentes a antibióticos.

keywords Antibiotic resistance; antibiotic resistance genes; microbial communities; hospital environment; wastewaters.

Abstract

Hospital environment is characterized by multiplicity of different bacterial species, which some of them can become resistant to antibacterial. Despite the specific nature of hospital effluents, they are considered to be the same pollutant and constituent load as urban wastewaters, being discharged to public sewage networks, collected and co-treated with the urban effluents in the WWTPs.

The aim of the study was to evaluate structure of microbial communities and to determinate the occurrence and abundance of genes associated with antibiotic resistance (*bla*$_{CTX-M}$, *bla*$_{VIM}$, *bla*$_{TEM}$, *bla*$_{SHV}$ and *intI*1) of Hospital wastewaters (hospital wards and microbiology laboratory) and urban effluents (pre- and post-treatment of WWTP of Cacia). DGGE and qPCR were performed.

The samples were collected in two different seasons, revealing differences in bacterial structure community. Hospitalizations and pre-and post-treatment communities were grouped in the some cluster, while microbiology laboratory communities were located in other cluster. The five genes selected were present in all effluents. The hospital wastewaters had the highest total abundance of the genes than the urban effluents. Of all genes *bla*$_{VIM}$ had a notable abundance due its high abundance in hospitalizations wastewater, followed by *intI*1. *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$ had the lowest abundances. Regarding with the biological treatment applied in the WWTP of Cacia, once the abundance of the genes was lower after the treatment, it is predictable that the treatment can be moderately effective.

Our results show that hospital settings tend to be an environment complex due the high microbial diversity of commensal and nosocomial pathogenic, the selective pressures created by antimicrobial use and the events of lateral gene transfer. The urban sewage network and the WWTP of Cacia represent a receptor of antimicrobial residues and antibiotic-resistance bacteria.

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List of Abbreviations

ARG	Antibiotic Resistance Gene
$CaCl_2$	Calcium Chloride
CIA	Chloroform-Isoamyl Alcohol
CS	Conserved segments
C_t	Threshold cycle
CTX-M	Cephalosporinase (cefotaximase)
DNA	Deoxyribonucleic Acid
D-Ala	D-alanine
DGGE	Denaturing Gradient Gel Electrophoresis
dNTPs	Deoxynucleotide triphosphates
DMSO	Dimethylsulfoxide
E	Reaction efficiency
EDTA	Ethylenediamine Tetraacetic Acid
HCL	Hydrochloric Acid
HGT	Horizontal Gene Transfer
HWW	Hospital Wastewaters
*Intl*1	Integrase 1
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LA	Luria Bertani Agar
LB	Luria Bertani Broth
LOD	Limit of detection
MDR	Multidrug-resistant
$MgCl_2$	Magnesium Chloride
MGE	Mobile Genetic Element
NaCl	Sodium Chloride
NAG	N-acetylglucosamine
NAM	N-acetyl-muramic
ORFs	Open riding frames

PBP	Penicillin-binding proteins
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
R^2	Correlation coefficient
SHV	Penicillinase (contraction of sulfhydryl variable)
SIMRIA	Sistema Municipal de Saneamento da Ria de Aveiro
s.d.	Standard deviation
UV	Ultraviolet
VR	Variable region
VGT	Vertical Gene Transfer
VIM	Verona imipenemase
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
TEM	Penicillinase (contraction of Temoniera)
WWTP	Wastewater Treatment Plant
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
6-APA	6-aminopenicillanic acid

1. Introduction

There are millions of species in the prokaryote domains, *Bacteria* and *Archaea*. Several studies related with the quantification of abundance and diversity of *Bacteria* and *Archaea* in many different natural environments have been reported. Aquatic environments appear to support less diversity than soils and sediments. The number of microbial cells in terrestrial ecosystems has been estimated around $4–5 \times 10^{30}$ microbial cells, while in water environments, such as oceans, the number of microbial cells is close to 1.2×10^{29} [(1)]. Thus, microorganisms represent a highly diverse group and constitute about 60% of the Earth's biomass. Microorganisms are in the heart of all ecosystems and play critical roles in modulating global biogeochemical cycles and influence all lives on Earth [(2,3)].

Beyond the studies reported for the elevated numbers and diversity of microorganisms, prokaryotes are recognized by their extremely diverse metabolic features, allowing them to adapt and inhabit in different environments. They have been described in habitats with normal conditions for their development, such as lake, rivers, soils, oceans and living organisms, but they are also found in extreme environments where temperatures, pH, radiation, salinity, pressure and oxygen tension can be even too high or too low [(4–6)]. Prokaryotes communities are extremely complex and structured entities characterized by their abundance, ubiquity and diversity, in which several members are key players in significant ecological processes as soil structure formation, decomposition of organic matter, recycling of essential elements and nutrients, regulation of atmosphere and photosynthesis [(6,7)].

Aquatic ecosystems represent habitats for interrelated and interacting microbial communities and populations of plants and animals. The aquatic environment plays therefore vital roles for ecosystem functioning, human health and civilization. It is also known that anthropogenic activities can lead to the conception of the urban water cycle, affecting different sectors such as waste, surface and drinking water, where bacteria can be exchanged and mobilized between different aquatic habitats (wastewater, disinfected

water and drinking water), other environments and ultimately, can even reach humans. Freshwater habitats are one of the most natural habitats with a rich bacterial diversity [8].

Hospital settings are characterized by multiplicity of different bacterial species, some of which may become resistant to antibacterial agents. This ecosystem remains complex, including not only the skin, gut and nasal flora of the patients, visitors and health professionals, as well as the environmental bacteria in everything from the water supply and drainage to bedside equipment and surfaces around. This means that one natural niche for a high diversity of microorganisms lies in the ecology of clinical settings. At the same time, this population of resistant commensal and environmental bacteria represents a reservoir for infections and dissemination of resistance, showing the ability to spread both human and natural environments [9–11].

In recent years, increasing attention has been paid to the presence of emerging pollutants, active principles of drugs and their metabolites, chemicals, micropollutants and even pathogenic bacteria in the effluents of Wastewater Treatment Plant (WWTP) and in surfacewater. Despite the specific nature of hospital effluents, they are frequently discharged to public sewage networks, collected and co-treated with the urban effluents in the WWTP [12,13].

1.1 Hospital Environment

Hospitals are colonized by a wide diversity of bacterial species and represent an excellent environment for the evolution and selection of antibiotic resistance. Although antibiotic resistance occurs due to the evolutionary natural selection, the misuse and overuse of antibiotics is dramatically increasing the selection of pathogenic bacteria resistant to multiple drugs. When these compounds are administrated wrongly in human or animal medicine, even for a short time, or small doses at inadequate strengths or for a wrong disease, bacteria are not killed and can acquire resistance genes and survive in the presence of these compounds, resulting in stronger infections and even death [14,15].

Bacteria have the ability to develop or acquire a remarkable array of mechanisms that enable them to survive in the hospital surfaces and to overcome the effects of antibiotic compounds. This permanence in the hospital environment results in a serious problem: these strains can easily infect immunocompromised patients and the surrounding individuals, and therefore can be in the origin of nosocomial infections and in some cases can be a cause of nosocomial outbreaks. Consequently, the emergence of multidrug-resistance (MDR) bacteria associated with infections is increasing, due to antibiotic selective pressure in the hospital environment [16].

These health-care associated pathogens include norovirus, *Clostridium difficile*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), Gram-negative bacilli as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [11,17]. These nosocomial pathogens are responsible for causing Hospital-acquired infections (HAIs) and they have two unremarkable properties: they are recognized as hospital pathogens and they have an innate ability to survive colonize and persist on dry inanimate surfaces, such as bed rails, bedside tables, surfaces of ventilators, sinks, suction equipment, mattresses, resuscitation equipment, curtains, slings for patient lifting, mops, buckets, door handles, stethoscopes, incubators, computer keyboards, respiratory tract equipment and devices, for long periods of time [17,18]. Therefore, contaminated

inanimate objects within the hospital environment can facilitate transmission of these nosocomial pathogens [19].

Patients colonized or infected with nosocomial pathogens transmit and contact those microorganism onto their clothes, bed, skin and environmental surfaces around. The most susceptible individuals can acquire pathogens through direct contact with the equipment and surfaces, or even through hand contact of health professionals [11].

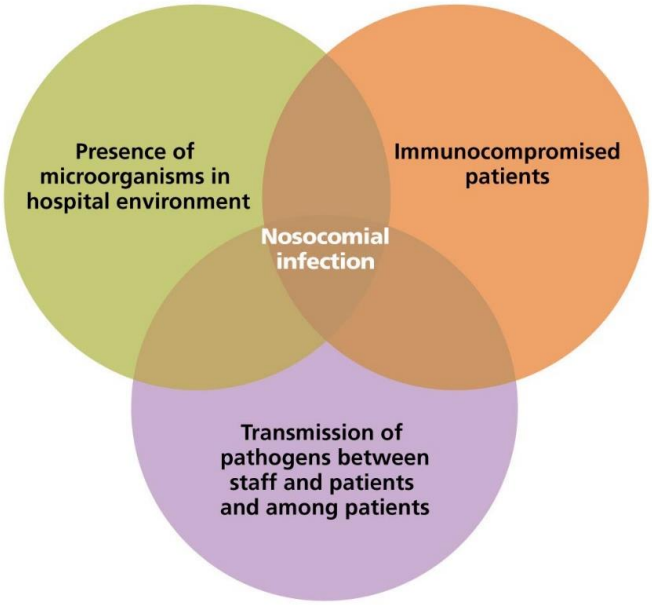


Figure 1: The interplay of factors resulting in nosocomial infections (http://images.google.com)

The overuse and misuse of antibiotics are important key points fueling this situation. The relationship between antibiotic use and emergence of resistance is complex [20]. These clinical settings constitute the main places where antibiotics are used and applied in large scale. However, antibiotics compounds are chemically stable and can remain active in natural environments even for a few years, allowing these MDR strains to be widely spread in the environment [21–23]. In addition, it has been reported that once antibiotics are administrated, the active substances of medicaments are metabolized, but only to some extent. The non-metabolized active substances are excreted by the patients in urine and in faeces, and enter into hospital effluents, which can be discharged into the urban

wastewaters without preliminary treatments, being co-treated with the urban wastewaters [13,14,24].

1.1.1 Hospital Effluents

Hospital wastewater (HWW) constitutes a major discharge of chemical residues and pharmaceuticals are found in all WWTP effluents, due to their inefficient removal by the conventional treatment processes [25]. Despite hospitals require a significant amount of water per day and for the different services, the quantity of wastewaters produced in a hospital depends on many factor: beds numbers, hospital age, water accessibility, general services inside the structure (laundry, kitchen), number and type of wards and units, institution management policies and others (**Figure 2**) [13].

HWW is usually discharged directly without pre-treatment. Although the HWW represent only a small portion of the total wastewater volume in the influent of a WWTP, the public and scientific attention to HWW is increasing in the last decade [26]. It is often assumed that hospitals are the most important source for input of antibiotics and resistant bacteria into municipal wastewater [27].

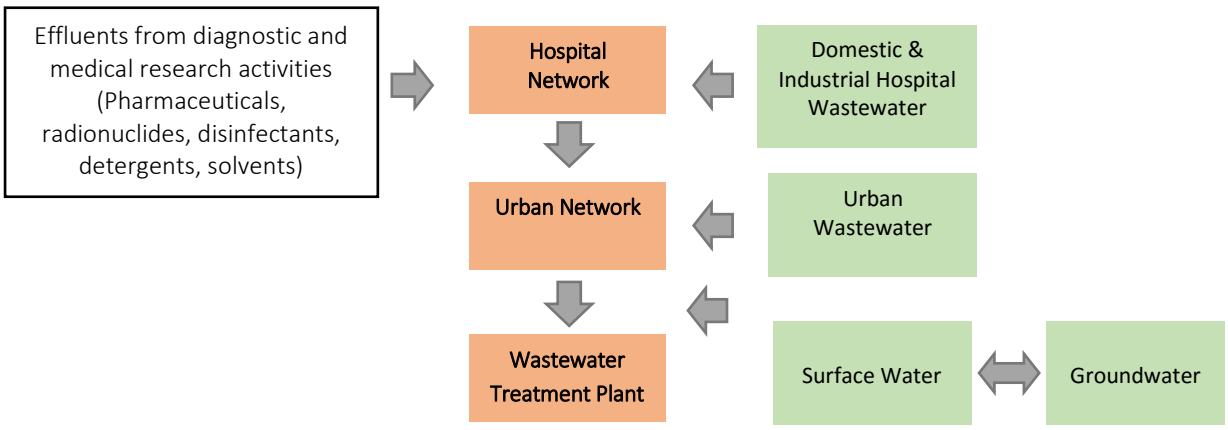


Figure 2: HWW problems and their impact on WWTP and natural environments (adapted from Emmanuel, Perrodin, Keck, Blanchard, & Vermande, 2005)

The common co-treatment of hospital and urban wastewaters at a municipal WWTP is not considered an appropriated practice, once it is grounded on dilution of different discharges and does not provide the separation of pollutants. Compared to urban wastewaters, HWW contain higher concentrations of pharmaceuticals, antibiotics, analgesics and bacteria. The concentrations of micropollutants in HWWs are between 4 and 150 times higher than in urban wastewaters [13].

The complications of removing micropollutants such as pharmaceuticals and bacteria from wastewaters are related to their concentration in the range of 10^{-3} - 10^{-6} mgL^{-1}, which is much smaller than the concentrations of the conventional macropollutants. The removal of the micropollutants is depended of their behaviour and fate in the WWTP (solubility, volatility, biodegradability, polarity and stability) and the conventional treatments are not designed to be able to remove greatly microcontaminants [13,25]. Some studies suggest that a pre-treatment of HWW prior to discharge into the sewers provides a reasonable solution and that this separate treatment may reduce the development of resistant bacteria, such as the amount of chemical and undesirable compounds [25–27].

1.2 Aquatic Environments

Water is one of the most important bacterial habitats on Earth, playing an important role for life and for human activities, reason why it represents an essential element for human existence. Water for human consumption is often subjected to direct or indirect contamination by different ways of pollution including microbial agents from sewage or contaminants from other origins. Simultaneously, the concern related with the risks of the public supply of water is increasing and it requires a most rigorous quality control and a use of more sophisticated analytical capabilities in order to facilitate the detection and quantification of physical, chemical and microbiological agents that are potentially hazardous to health. For a long time that the presence of microorganisms from faecal materials in drinking or recreational water is recognized as a source of infectious diseases caused by bacteria, viruses or protozoa, representing a risk to human health [28,29].

Water is an ideal medium for bacterial life, affording access to readily accessible dissolved nutrients, as well as protection from desiccation and UV light [30]. From all the microbial agents present in water, there are exogenous microorganisms (from faecal matters) and the indigenous microorganisms (which belong to the natural water microbiota). Many of the pathogens associated to water can be also transmitted by food, which become more difficult to determinate the origin of some sporadic infections. Unless there is a failure in the water treatment process, normally an inadequate disinfection or a high intake of contaminated water, then the occurrence of pathogenic bacteria as *Salmonella*, *Shigella*, *Vibrio* and *Campylobacter* are rarely found. However, a wide range of opportunistic pathogens as *Aeromonas*, *Pseudomonas* and some species of *Mycobacterium* can be more commonly found [28,29].

After being used, water returns to the environment in two possible forms, partially polluted or totally polluted which may compromise the quality of available water resources and increase the risk of waterborne diseases transmission. This pollution can occur due to the transport of toxic or chemical substances, organic materials and pathogenic microorganisms [28].

1.2.1 Wastewater Treatment Plants

The systems of water treatment are processes performed in untreated water in order to get a potable, chemical, bacteriological and biologically safe product for human consumption. For this, it is extremely necessary to remove and destroy any harmful organisms, harmful chemical substances as other materials either in suspension or solution. The type of water treatment differs with its origin (surface or underground) and its initial quality. Thus, the treatment of water for human consumptions means the production of healthy water that respects the chemical and microbiological safety requirements. The diversity of water treatments comprises clarification and sedimentation, filtration and disinfection [25,31].

Wastewaters can be designed as a recombination of liquid or water-carried waste removed from residences, institutions, and domestic, commercial and industrial establishments, in association with ground, surface and storm water. Its composition includes high load of oxygen demanding wastes, pathogenic agents, organic materials, some nutrients which stimulates plant growth, inorganic chemicals and minerals, sediments and sometimes toxic compounds [31].

WWTPs are potential hot spots for horizontal transfer and selection of antimicrobial resistance genes among aquatic bacteria due the high microbial biomass and the richness of nutrients [32,33]. Despite of representing a rich bacterial diversity and the high organic loads concentration that favor microbial growth, wastewater effluents suffer strong anthropogenic pressures as high levels of pollutant compounds (heavy metals, detergents, pesticides, antibiotics) [34].

Conventional wastewater treatments consists of a combination of physical, chemical and biological processes and operation to remove solids, organic matter and sometimes nutrients of the wastewaters [31].

Wastewater treatment can be differentiated as preliminary, primary, secondary and tertiary and/ or advanced wastewater treatment.

i. Preliminary treatment consists in the removal of coarse solids and other large materials often found in raw wastewater, which helps to remove or to reduce in size the large, entrained, suspended or floating solids (pieces of wood, cloth, paper, plastics, garbage, together with some fecal matter).

ii. Primary treatment consists in physical operations used to remove solids occurring in wastewater.

iii. Secondary treatment intends to treat the effluent from primary treatment in order to remove most of organic matter and suspended solids with the use of biological and chemical processes.

iv. Tertiary treatment intends additional processes and advanced biological methods, which are used to remove constituents namely, nitrogen or phosphorus that are not

reduced by the secondary treatment. It is defined as any process designed to produce an effluent of higher quality than that achieved by a secondary treatment.

During the process of wastewater treatment, major qualitative and quantitative changes are observed in the distribution of the bacterial population [14,35].

1.3 β-lactams Antibiotics

Antibiotics are drugs used to treat bacterial infections that exert their action on bacteria by one of two basic ways. Some classes of antibiotics are classified as bacteriostatic, because they can act on the bacterial cell, inhibiting their growth or cell division; while others are bactericidal and may act on the bacterial cell leading to death [36]. Some antibiotics have bacteriostatic activity in some circumstances while in other situations have bactericidal activity. Meanwhile, antibiotics can develop their action against a limited variety of bacterial species – narrow spectrum antibiotics; or can be efficient against a wide range of pathogens of different species – broad-spectrum antibiotics [37]. These compounds can be classified according to their chemical structure, action (therapeutic use) and spectrum of activity. Antibiotics of the same class have a similar toxicity and spectrum of activity [38]. They can be natural or chemical products synthesized in laboratory and designed to selectively block essential processes in bacterial cells (eg, sulfonamides and quinolones) [39].

The β-lactams antibiotics have been largely used as therapeutic agents since the second world war [40]. The β-lactams antibiotics represent the group of antibacterial agents more diverse and used in the clinical treatment of infections caused by various bacteria, either in hospital or in the middle clinic [41,42].

The β-lactam family is characterized by the presence of a β-lactam ring, cyclic structure of four atoms (one of carbon and three of nitrogen) with radical substituents. The β-lactam ring is necessary for antibacterial activity, and the side chain determines the antibacterial

spectrum and pharmacologic properties. Depending on the structural characteristics of the side-chain β-lactams are classified into penicillins, cephalosporins, monobactams, carbapenems and inhibitors of β-lactamases (**Table 1**) [41,43]. A short description is given below:

A. **Penicillins and derivatives:** reduced spectrum activity and susceptible to hydrolysis by the bacterial enzymes β-lactamases. The development of new molecules derived from the core compound of penicillin, 6-aminopenicillanic acid (6-APA), make it possible to obtain semi-synthetic penicillins [43].

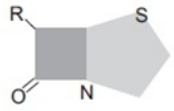
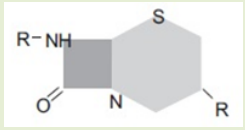
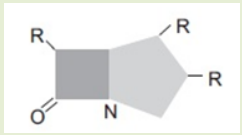
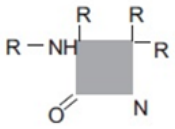
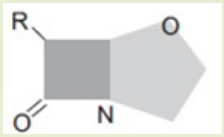
B. **Cephalosporins:** Semi-synthetic antibiotics grouped in first, second, third, and forth generation cephalosporins according to their spectrum of activity and to the timing of introduction in therapeutics [44].

C. **Carbapenems:** considered as broad spectrum antibiotics. Imipenem and meropenem are well known representatives [41].

D. **Monobactams:** The β-lactam is alone and not combined to another ring. Although they do not contain a nucleus with a fused ring attached, they belong to β-lactam antibiotics [45].

E. **β-lactam inhibitors:** These compounds, like clavulanic acid, exhibit negligible antimicrobial activity and are usually combined with other β-lactams to combat strains that express β-lactamases [43].

Table 1: Classification of β-lactam antibiotics (adapted from Samaha-Kfoury & Araj, 2003; Suárez & Gudiol, 2009)

β-lactams Groups	Chemical structure	Examples of β-lactam antibiotics
Penicillins		Natural: Penicillin G, Penicillin
		Penicillinase resistant penicillins: methicillin, nafcillin, oxacillin, cloxacillin
		Aminopenicillins: ampicillin, amoxicillin
		Carboxypenicillins: carbenicillin, ticarcillin
		Ureidopenicillins: mezlocillin, piperacillin
Cephalosporins		First generation: cefazolin, cephalothin, cephalexin
		Second generation: cefuroxime, cefaclor, cefamandole, cefamycins (cefotetan, cefoxitin)
		Third generation: cefotaxime, ceftriaxone, cefpodoxime, ceftizoxime, cefoperazone, ceftazidime
		Fourth generation: cefepime, cefpirome
Carbapenems		Imipenem, meropenem, ertapenem
Monobactams		Aztreonam
Inhibitors of β-lactamases		Tazobactam, Clavulanic Acid

1.3.1 Mode of Action of β-lactam Antibiotics

Many of the antibiotics which act on the cell wall were discovered before the understanding of the biochemistry of cell wall biosynthesis [46]. The cell wall is responsible for maintaining the bacterial shape and its integrity has a critical importance to cell viability. The cell wall provides an effective mechanical protection, which prevents the osmotic rupture of the cell in a hypotonic environment. It is composed by a dynamic structure that is continuously synthetized, modified and hydrolyzed in order to allow the cell growth and division [47,48]. If the peptidoglycan synthesis is inhibited, it results in the death of a growing bacteria [49].

Despite the differences related to the structure profile and the chemical composition of the cell wall of Gram-negative and Gram-positive bacteria, the scaffold of the cell wall consists of the cross-linked polymer called peptidoglycan (murein), a macromolecule which surrounds the bacterial cell and it is only found in prokaryotic cells. It is a molecule composed of amino acids and sugars, being constituted by parallel linear chains of two amino sugars, N-acetylglucosamine (NAG) and N-acetyl-muramic (NAM) disposed alternately and joined by β1-4 glycosidic linkages [48,50].

Into each NAM molecule the peptide chains composed by modified amino acids are linked by amide bonds. The peptide chains of the surrounding linear chains establish among themselves inter-peptide bonds (crosslinking). This transpeptidation is the final step in peptidoglycan biosynthesis, and confers resistance to the bacterial cell wall, representing important natural target of β-lactam antibiotics, including penicillins and cephalosporins. The polymerization of the glycan strand (transglycosylation), and the cross-linking between glycan chains (transpeptidation) are catalyzed by the Penicillin-binding proteins (PBPs). These enzymes are transpeptidases or carboxypeptidases involved in peptidoglycan metabolism [51,52].

While the peptidoglycan is being synthetized and during the reaction between two close chains, it must occur the acylation of a Ser residue present in the catalytic-site (active-

site) of PBPs. Then, once the complex enzyme-D-Ala-D-Ala of a peptidoglycan molecule is created, the catalytic process is concluded through the ligation to a peptide chain of a second residue and this process allows the creation of the peptidoglycan structure [50,51].

On the contrary, β-lactam antibiotics have slow activity as antibacterial, inhibiting the synthesis of cell wall and causing lysis of the bacteria [41]. Firstly, they bind to the bacteria cell wall and then, they work by blocking the final stage of peptidoglycan synthesis through the inhibition of the enzymes involved in its formation (transpeptidases, carboxypeptidases). This mechanism is based on the binding of β-lactam antibiotics to the PBPs, preventing the synthesis of an intact cell wall. These antibiotics are only active against bacterial cells growing. The affinity of the antibiotic to PBPs is explained by the fact that the structure of the amide bond of the β-lactam ring is analogous to peptide bond of the D-alanyl-D-alanine [41,47].

Figure 3 shows a schematic representation describing the cell wall formation synthesis, as well as the mechanism of action of β-lactam antibiotic.

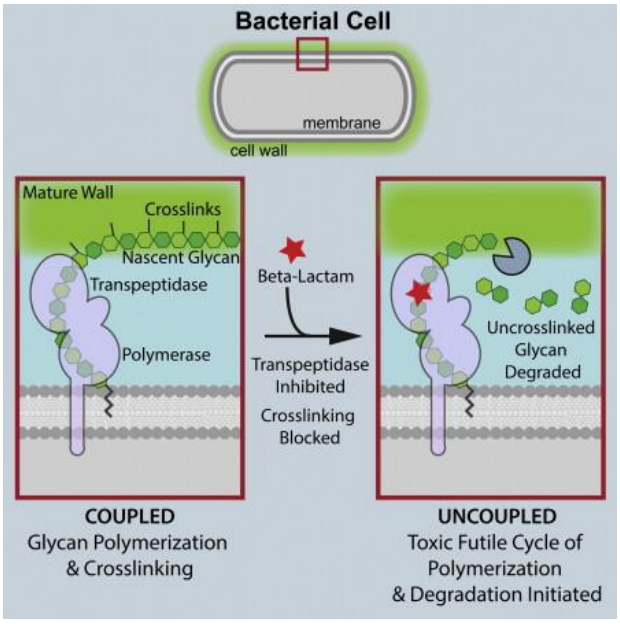


Figure 3: Cell wall formation and mechanism of action of β-lactam antibiotics (Cho, Uehara, & Bernhardt, 2014)

1.3.2 Resistance Mechanisms associated with β-lactam antibiotics

Antibiotics reduce the bacterial grow or shut down essential cellular functions. Bacteria have the ability to develop different resistance strategies in order to adapt to the changes in their surroundings preventing antimicrobial agent from reach its target and acquire different mechanisms to render ineffective the antibiotics used against them [38,53]. The antibacterial efficiency of a β-lactam antibiotic depends on the accessibility to its targets or the ability to diffuse through the cell membrane, the stability to bacterial hydrolytic enzymes (β-lactamases) and the affinity for the target enzymes (PBP's) [54].

Like it was mentioned before, the overuse/misuse of β-lactam antibiotics during the last years has largely contributed to the improvement, by bacteria, of several resistance mechanisms to these antibiotics. Four types of β-lactam resistance mechanisms are described as in **Figure 4**: (i) reduction of the permeability of outer membrane, (ii) alteration of the antibiotic target site (the PBPs), (iii) development of effective efflux systems and (iv) enzymatic degradation of the antibiotic mediated by β-lactamases. The hydrolysis of the β-lactam ring by beta-lactamases represents the most common and efficient resistance mechanism, subject that will be explored in **section 1.4** [55].

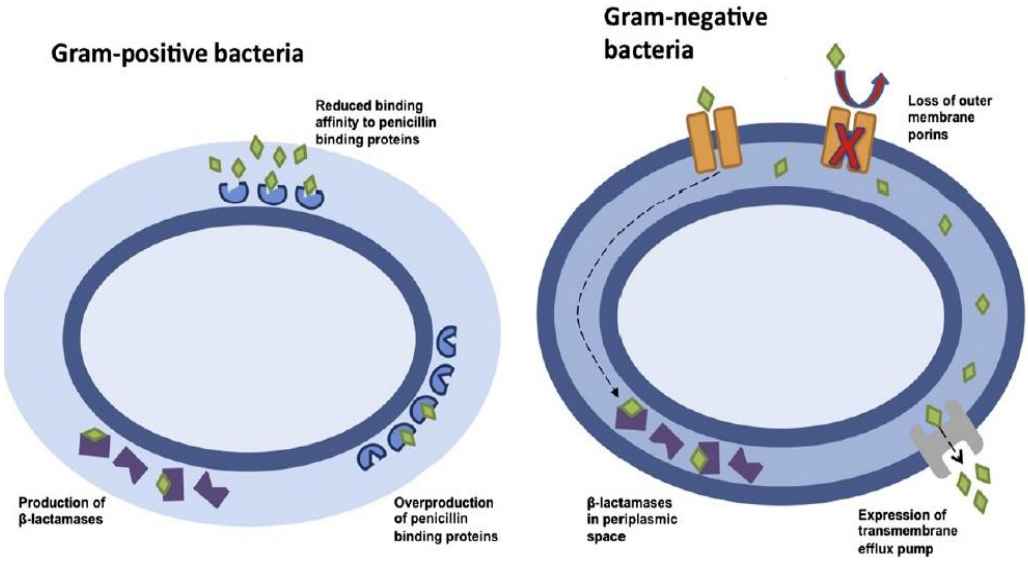


Figure 4: Major mechanisms of β-lactam resistance in both Gram-positive and Gram-negative bacteria (Tang et al., 2014)

i. Reduction of the permeability of outer membrane

The cell wall of Gram-negative bacteria differs from the wall of Gram-positive bacteria, for instance by the presence of an outer membrane [55]. This barrier performs a crucial role by offering protection from harmful compounds in the extracellular environment and by ensuring the entrance of nutrients and substances (antibiotics) to the bacterial cell through the presence of specific proteins responsible for the formation of channels known as porins [56].

The diffusion of molecules and antibiotics is influenced by its charge, structure and size. Reductions in the cell permeability decrease the antibiotic flow and consequently its concentration in the periplasmatic space. Modifications in membrane permeability leading to the reduction of the antibiotic uptake across the outer membrane of Gram-negative bacteria [57]. One example of reduced cell permeability is related with strains of *Pseudomonas aeruginosa*, which have a reduced number of porins in the outer membrane and, therefore, they are intrinsically resistant to many antibiotics [56] .

Besides the reduced number of porins, the changes in the cell permeability can occur as a result of porins loss due to mutation. Among the main porins described in Pseudomonas aeruginosa, OprD porin has a biological function in the passive uptake of basic amino acids across the outer membrane, but it also forms pores that are permeable to carbapenems, like imipenem is extremely effective against this bacteria through its diffusion by this porin [56,58]. Another example is related to the largest and most abundant OprF porin, required for cell growth in low-osmolarity environment and for the maintenance of cell shape, and probably the most used by β-lactam antibiotics to penetrate within the bacteria [59]. In **Figure 5** are schematically represented some examples of antibiotic resistance mechanisms associated with porins.

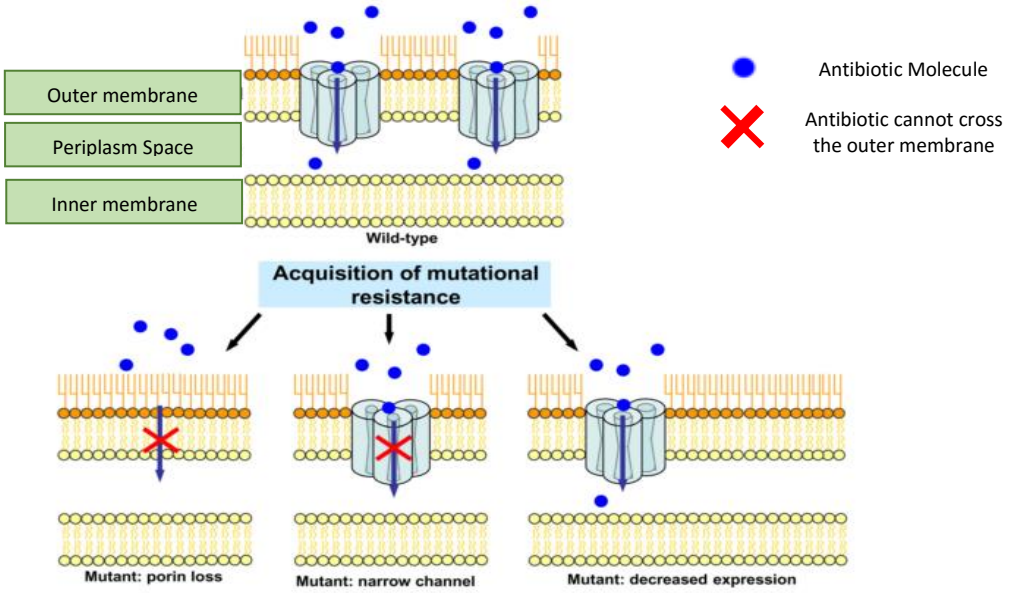


Figure 5: Examples of different mechanisms of acquisition of mutational resistance associated with porins (adapted from Fernández & Hancock, 2012)

ii. Alteration of the antibiotic target site

One of the mechanisms whereby bacteria become resistant to antibiotics emerges through changing of the antibiotic target site, the PBPs. In most cases, these transpeptidases become less susceptible to binding with the antibiotic. Although this fact has been observed in Gram-negative bacteria, this resistance mechanism is particularly evident in Gram-positive bacteria. The changes in PBPs, including the expressions of PBPs with reduced affinity for β-lactam antibiotics or the decreased expression of specific PBPs, avoids the establishment of covalent bonds between β-lactam and PBPs conferring resistance to these compounds [(60)].

The modification of these transpeptidases can be accomplished by one of the four mechanisms: acquisition of an additional low-affinity PBP; overexpression of an endogenous low-affinity PBP, increasing the level of resistance to β-lactam antibiotics; alteration of endogenous PBPs by point mutations, promoting the reduction of the affinity between PBP and the antibiotic or by homologous recombination or a combination of the above [(52)].

iii. Development of effective efflux systems

In order to prevent the intracellular accumulation of antibiotics, bacteria have developed energy-dependent systems to pump such compounds out of the cell, providing an effective mechanism of resistance in some bacterial strains [61]. Efflux pumps are normally proteins located in the cytoplasmic membrane, responsible for the extrusion of the antibiotic from cells into the external environment. These proteins are found in both Gram-positive and -negative bacteria [62].

In Gram-positive bacteria, which lack the outer membrane, the cell wall does not offer much resistance to diffusion of small molecules and efflux pumps always include a single polypeptide located in the cytoplasmic membrane [61].

However these efflux systems present a major complexity in Gram-negative bacteria due to the outer membrane, which acts as an additional protective barrier limiting the penetration of the antibiotic molecules. The antibiotic crosses the outer membrane and once the antibiotic reaches the cytoplasm, it can be picked up in the periplasm and directly expelled to the external medium. In this group of bacteria, resistance mechanisms appear often associated with changings in membrane permeability as the increased synthesis of constituent proteins of the efflux pump, due, for example, to mutations that occur in the repressor genes of these proteins. These mutations can also lead to the increased efficiency of the antibiotic transport to the outside of the cell. This mechanism can also appear associated with the production of β-lactamases [63,64].

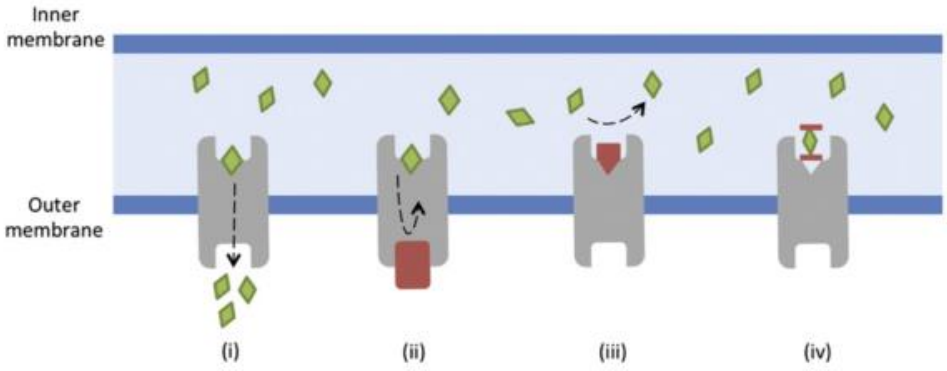


Figure 6: Bacterial efflux pump (Tang, Apisarnthanarak & Hsu, 2014)

In **Figure 6** is represented the effects of efflux pumps systems. In (i), it is described the extrusion of antibiotic molecules. In (ii), efflux pump inhibitor plug is blocking the outer membrane channel of the efflux system. In (iii), a non-antibiotic molecule or an efflux pump inhibitor is binding the site. In (iv) the antibiotic molecule is modified in order to avoid the extrusion by the efflux pump [60].

(iv) Enzymatic degradation of the antibiotic mediated by β-lactamases

The most common mechanism is the production of enzymes responsible for the degradation or modification of the antibiotic before it can reach the intended target, generating ineffective products. Therefore, the β-lactamase family degrades β-lactam antibiotics and are found widely in both Gram-negative and Gram-positive bacteria [42,65]. These bacterial enzymes inactivate β-lactam antibiotics by hydrolytic cleavage of the β-lactam ring, and their synthesis can be either chromosomal which can be inherent to the organism or plasmid mediated. As example, *Pseudomonas aeruginosa* possess chromosomal β-lactamase and often also acquires genes encoding β-lactamase which may be located in plasmids [42,66].

The spread of β-lactamase genes has been largely accelerated by their combination with mobile genetic elements, as plasmids or integrons, allowing the transfer of genetic material to different bacteria. In Gram-positive bacteria, these hydrolytic enzymes are excreted as exoenzymes to the extracellular environment. In Gram-negative bacteria, β-lactamases are secreted to the periplasm space and remain bounded to the cytoplasmic membrane, where they will degrade the β-lactam antibiotic before it can interact its receptor site [42,65].

Given the diversity of enzymatic features of many β-lactamases, as their structure and substrates profiles described so far, many attempts were made to classify these enzymes [67]. There two major approaches to categorize the β-lactamases family are represented in **Table 2** and they are:

i. **Molecular Classification:** based in the molecular structure of the enzyme like the nucleotide and amino acid sequences, proposed by Ambler in 1980. The β-lactamases are divided into four molecular classes (A to D). Classes A, C and D are serine-dependent enzymes that catalyze the hydrolysis of β-lactam antibiotics via a serine-bound formed between the substrate and the active site serine. Class B or metallo-β-lactamases are enzymes that require zinc for their β-lactam catalysis [49,65,68]. As in the earlier functional classifications, enzymes were aligned based on their ability to hydrolyze specific β-lactam classes and on the inactivation properties of the β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam. A description of each of the functional groups follows.

ii. **Functional Classification:** focused in the functional and biochemical features, for example the antimicrobial substrate profile (their ability to hydrolyze specific β-lactam classes and enzyme inhibition profile (the inactivation properties of the β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam). According with this methodology, the first step is based on enzyme inhibition by EDTA chelating agent and the β-lactamases inhibited by this chelant are categorized in group 3 (metallo-β-lactamases). After this evaluation focused in the enzyme inhibition, the enzymes are grouped according with the substrate profile. If the enzymes are able to hydrolyze benzylpenicillin and cephaloridine, they are considered as penicillinase or cephalosporinase, being included in group 1 and 2. The other subsets are defined due to their hydrolysis levels to other antibiotics. The inhibitors profile is also important, particularly for the β-lactamases that are not inhibited by clavulanic acid, which allowed the classification of these β-lactamases into group 4 [42,69].

Table 2: Groups proposed by the Bush classification and relationship with the Ambler classification (Adapted from Dhillon & Clark, 2011 and from Bush & Jacoby, 2010)

Functional Group	Molecular Class	Common name	β-Lactams to which resistance is conferred
1	C	Cephalosporinase	Penicillins, cephalosporins
2b	A	Penicillinase	Penicillins, early cephalosporins
2be	A	Extended-spectrum β-lactamase	Penicillins, extended-spectrum cephalosporins, monobactams
2d	D	Cloxacillinase	Penicillins, including oxacillin and cloxacillin
2df	D	Carbapenemase	Carbapenems and other β-Lactams
2f	A	Carbapenemase	All current β-Lactams
3	D	Metallo-β-lactamase	All β-Lactams except monobactams
4	-	Penicillinase	Penicillins

1.4 Resistance Determinants approached in this study

Resistance determinants can be inherited by vertical gene transfer (VGT), when they pass through generations as the cells divide as represented in **Figure 7**.

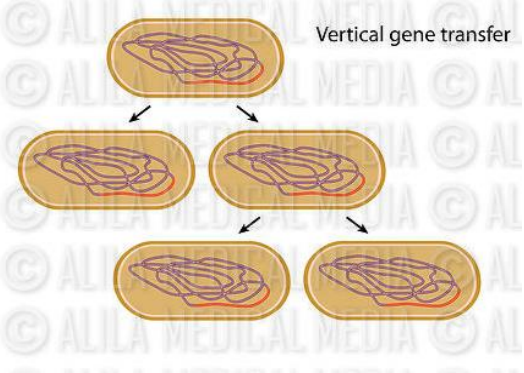


Figure 7: Representation of the vertical gene transfer (http://images.google.com)

Resistance determinants can be also acquired by horizontal gene transfer (HGT), which involves the movement of genetic information between strains of bacterial species or genera [70]. The HGT is associated with mobile genetic elements (MGEs). HGT occurs through three different processes: via transformation (release of DNA by lysis and uptake by competent recipients), transduction (with the help of a bacterial virus) and conjugation (direct cell-to-cell contact for transfer of extra-chromosomal or chromosomal DNA) (**Figure 8**) [71].

Antibiotic resistance genes (ARGs) are associated to MGEs like conjugative plasmids, transposons, insertion sequences (IS), integrons and bacteriophages, which can transport them in tandem [23,70]. MGEs promote the movement of DNA within or between genomes, having a significant evolutionary impact and an important role in the dissemination of ARGs, once they allow the quick adaptation to the antibiotic's classes in the environment and consequently, increasing the rate of MDR and their survival [38]. HGT plays a critical role in the resistance dissemination and by the study of the resistance determinants' sources and the MGEs that spread them, it is possible to predict future threats and to try to control future recruitment and spread of ARGs [72].

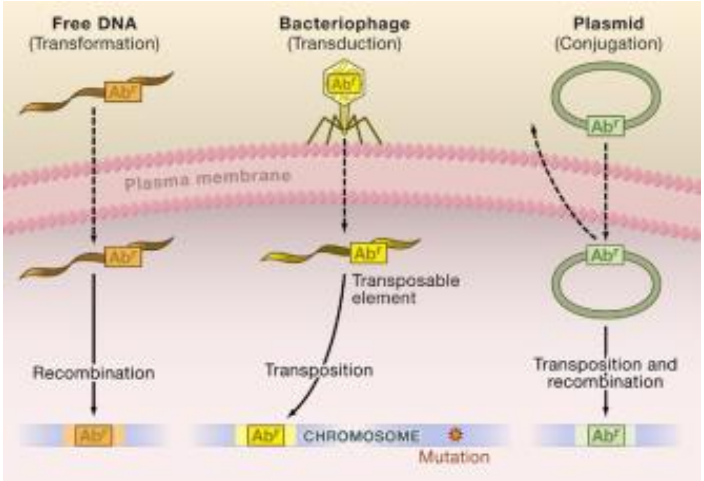


Figure 8: Mechanisms of horizontal gene transfer (Alekshun & Levy, 2007)

In this study, we focused our attention on *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{VIM}$ and class 1 integron integrase (*Int*1) genes.

1.4.1 Extended-spectrum β-lactamase (ESBL)

The first extended-spectrum β-lactamase (ESBL) was identified in an isolated *Enterobacteriaceae* in Germany in 1983, after the introduction of broad spectrum cephalosporins. Since then, the incidence of ESBLs has been increasing worldwide, being mostly described in *Escherichia coli* and *Klebsiella* [73,74]. There is no consensus of the exact designation of ESBL and a general definition consists of several elements [75,76].

Bacteria producers of ESBL has an important role in the global spread of resistance in hospital environments. Many factors as the increased period of hospital permanence, insertion of catheters, execution of invasive procedures, chirurgic interventions or implementation of renal replacement therapies have been widely associated to the isolation of bacteria producers of ESBL in hospitalized patients [77,78].

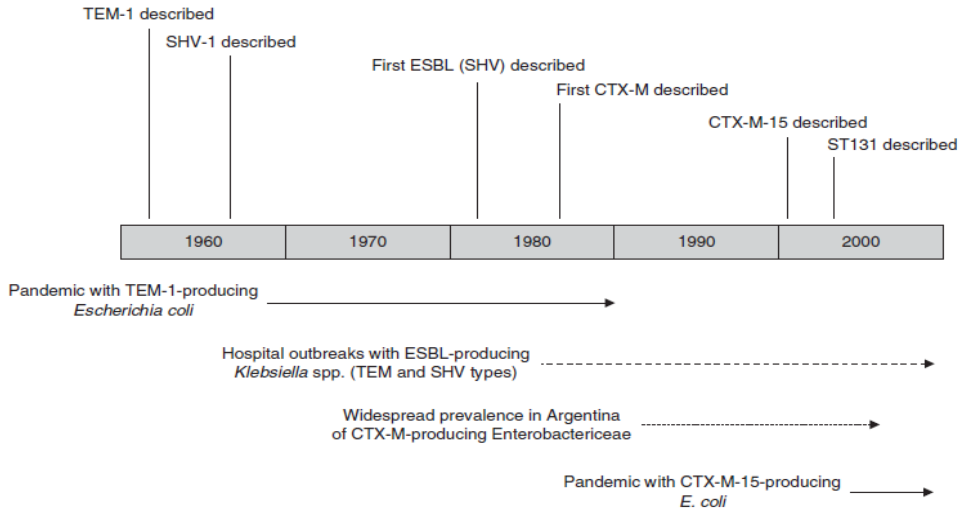


Figure 9: Recent evolution of pandemics caused by bacteria that produce β-lactamases, especially extended-spectrum β-lactamases (ESBL) (Pitout, 2010)

These enzymes are classified in class A and D (Ambler classification) or in the subgroup 2be or 2d (functional classification). Their expression is associated with bacterial resistance to penicillins, first-, second-, and third-generation cephalosporins and are inhibited by β-lactamase inhibitors as clavulanic acid [79]. There are various genotypes of ESBL and they are classified in different ways according to their amino-acid sequence and to their hydrolytic profile. The most common are SHV, TEM and CTX-M [66,80]. Most of ESBL derived from simple point mutations (substitution of a single amino acid) in the active site of the classical β-lactamases TEM-1, TEM-2 and SHV [75]. So far, there are several variants of TEM and SHV types, although not all of them have ESBL phenotype. Unlike TEM/SHV enzymes, all the CTX-M enzymes are ESBLs [81].

i. **TEM β-lactamases**: The first β-lactamase identified, plasmid-mediated in gram-negative, was TEM-1 and it was discovered in Greece in 1960 from an *E. coli* isolated from a patient called Temoniera, giving the designation of TEM β-lactamase [80]. TEM-1 is capable to hydrolyze penicillins and early cephalosporins (cephalothin and cephaloridine) [80,82]. The amino acid substitutions in TEM-1 occur in a limited number of positions. Combinations of these substitutions result in various phenotypes of ESBLs as the ability to hydrolyze cephalosporins such as ceftazidime and cefotaxime [80]. The number and position of the amino acid replacements are very important for the ESBL phenotype production. The substitutions of glutamate by lysine in position 104, arginine by histidine or serine at position 164, glycine by serine at position 238 and glutamate by lysine at position 240 are the most important [80]. Although the β-lactamases TEM type are frequently found in *Escherichia coli* and *Klebsiella pneumoniae*, they also have been reported in many different species for example of the family *Enterobacteriaceae, Pseudomonas aeruginosa, Haemophilus influenzae* and *Neisseria gonorrhoeae* [76,80].

ii. **SHV β-lactamase**: Meanwhile, another β-lactamase, known as SHV-1 (because sulfhydryl reagents had a variable effect on substrate specificity) and sharing 68% similarity in terms of amino acid sequence with TEM-1, was found in *Klebsiella*

pneumoniae, *E. coli* and wide range of *Enterobacteriaceae* [79]. The SHV-1 β-lactamase is chromosomally encoded in the majority of isolates of *K. pneumoniae*, but it is usually plasmid mediated in *E. coli* [80,82,83]. Most SHV are characterized by substitution of a glycine for serine at position 238. The changes that have been observed in bla_{SHV} to give SHV variants occur in less different positions [80]. SHV-1 confers resistance to broad-spectrum penicillins as ampicillin, tigecycline and piperacillin but not to the oxyimino substituted cephalosporins [82].

iii. **CTX-M β-lactamase**: CTX-M β-lactamases represent a new family that specially hydrolyzes cefotaxime and that have emerged and spread during recent years. It has been frequently been detected in isolates of *E. coli*, *Salmonella enterica* serovar *Typhimurium* and other species of *Enterobacteriaceae*. The CTX-M β-lactamases are encoded by genes that have been captured by mobile elements from the chromosome of the environmental bacteria *Kluyvera* spp. [73,80]. This family has been first described in Germany and Argentina, and they are not very closely related to TEM or SHV β-lactamases, showing only 40% of identity with these β-lactamases [78,80]. Another feature that characterizes these enzymes is related with the fact that they are better inhibited by the β-lactamase inhibitor Tazobactam than by sulfabactam and clavunate. It includes five different groups of enzymes (CTXM-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25) [76,78,80,84]. So far, there are more than 200 CTX-M variants [85].

1.4.2 Metallo-β-lactamases

In the mid-1960s MBL were firstly detected in species with low pathogenic potential. Since the 1990s, several of these enzymes encoded by mobile DNA elements have emerged in important Gram-negative pathogenic bacteria as Enterobacteriaceae, *Pseudomonas aeruginosa,* and *Acinetobacter baumannii*, increasing their clinical relevance [86]. MBL belong to class B of the structural classification of β-lactamases [67]. They are considered metallo-enzymes since they require the presence of one or two zinc

cations in the active site and are inhibited by the chelating agent EDTA. These enzymes usually can confer resistance to some penicillins, cephalosporins and carbapenems, but not to monobactams [86,87].

The most important types for epidemiological dissemination and clinical relevance are the IMP-type, VIM-type, SPM-type, and NDM-type enzymes. The VIM-type enzymes (Veronese imipenemase) were discovered in Europe in 1990s and more than 20 VIM allotypes are described. These enzymes are known by their high affinity for carbapenems. VIM-1 was reported in *Pseudomonas aeruginosa* in Verona, Italy, followed by VIM-2 reported in Greece and France [86,88]. bla_{VIM} gene is horizontally movable because it is normally inserted in integrons and some of them are located in conjugative plasmids [86,89].

1.4.3 Class 1 integron integrase gene

Integrons are DNA elements that can be transferred from one organism to another, but they cannot move by themselves. They have the ability to capture genes that confer antibiotic resistance by site-specific recombination mechanism, instead of transposition [23,90]. They are gene expression elements capable of incorporate open reading frames (ORFs) and convert them to functional genes. These ORFs are contained in modular structures known as gene cassette which are the smallest genetic entities that can carry resistance determinants. Genes cassettes are not necessarily part of the integron, but they become part of the integron when they are integrated [91,92].

i. Integron structure and transferability

These DNA elements are characterized by two conserved segments (CS) flaking a central region known as variable region (VR) in which the gene cassette can be inserted [92,93]. They are composed of three keys elements necessary for to integrate and express exogenous genes: a gene encoding for an integrase (*intI*), a primary recombination site (*attI*) and a strong promoter that will promote the expression of any suitable integrated gene [91,94].

The integrase encoded by *int*I gene belong to a tyrosine recombinase family, which includes proteins that recombine DNA duplexes by performing two successive strand breakage and rejoining steps and a topoisomerization of the reactants. The integron integrase catalyze the insertion and excision of gene cassettes by specific-site recombination. Recombination takes place between two recombination sites, *attI* and *attC*. The integrase mediates recombination first between the *attI* site, located in the 5'-CS of the integron platform, downstream the *intI* gene where the incorporation of gene cassettes occurs, and a secondary target called an *attC* site. The *attC x attI* rearrangements catalyzed by integrase lead preferentially to cassette integration at *attI* site, allowing gene cassette expression. *attC x attI* recombination mediated by integrase may also occur, resulting in gene cassette deletion through circular intermediate [95–97].

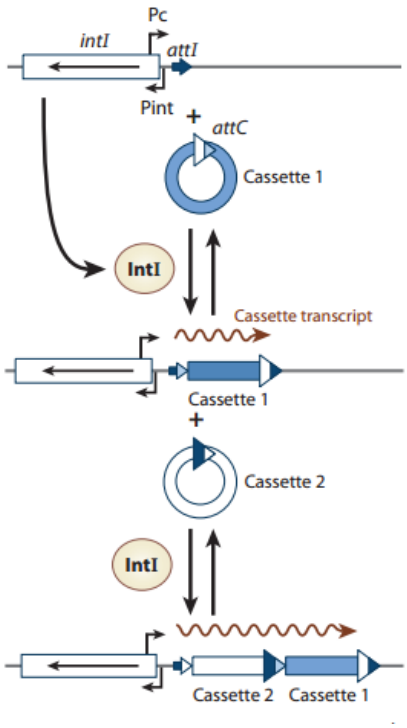


Figure 10: Mechanism of integron-mediated gene capture. Integrase catalyzes the insertion of circular gene cassettes at the *attI* site through site-specific recombination (Cambray, Guerout, & Mazel, 2010)

ii. Class 1 integrons

The class 1 integrons are frequently associated with plasmids or transposons, containing few gene cassettes that encode antibiotic resistance determinants, or can be located on the bacterial chromosome, in which case can contain several hundreds of cassettes encoding unknown functions [97,98]. These integrons have been distributed in clinical organisms [99,100].

2. Objectives

The following study was conducted in order to clarify the following aspects:

- Are there resistance genes in hospital effluents? Do these antibiotic resistant genes reach the municipal networks? Do they reach the environment after the wastewater treatment?
- What are the detectable differences between the structure of microbial communities from the hospital wastewaters and from the wastewaters from the treatment plant?
- In the WWTP, is there differences in the microbial communities before and after the treatment? Is there any change in the abundance of ARGs before and after the treatment?

To achieve this, the following approaches were employed/used:

i. To evaluate the presence of ARGs, such as *bla*$_{CTX-M}$, *bla*$_{VIM}$, *bla*$_{SHV}$, *bla*$_{TEM}$ and *int*I1 in bacterial communities inhabiting wastewaters with different origins;

ii. To perform the absolute quantification by quantitative PCR (qPCR) of the same ARGs mentioned before;

iii. To evaluate the composition and bacterial diversity of communities between the different samples from the Hospital Aveiro and WWTP Aveiro effluents, collected in two distinct seasons, summer and winter.

3. Material and Methods

3.1 Site description

The Centro Hospitalar of Baixo Vouga, E.P.E. – Aveiro represents an institution which goal is to provide the quality health care to a population of about 300000 inhabitants, spread over nine municipalities of Aveiro District. With 1500 employees, the hospital provides support in clinical services, 400 beds of hospitalization and two Emergency Services open 24 hours: the one from Aveiro (Medical-surgical emergency) and the other of Águeda (basic urgency). However, the clinical placement of this study was related with the facilities in Aveiro, more specifically the effluents of hospitalizations wards and of microbiology laboratory [(101)].

The WWTP of Cacia, located in Cacia (Aveiro), was designed to satisfy the treatment needs of effluents from Águeda, Aveiro, Albergaria-a-Velha, Estarreja, Murtosa, Oliveira do Bairro, Ovar and Santa Maria da Feira. This WWTP is part of Saneamento Integrado dos Municípios da Ria de Aveiro (SIMRIA), which is responsible for the construction, management and exploration of Sistema Multimunicipal de Saneamento da Ria de Aveiro. The main goal of SIMRIA is related with the satisfaction of collection needs, treatment and rejection of domestic and industrial wastewater effluents from the municipalities mentioned above (http://www.adra.pt/). This WWTP execute a biological treatment of secondary level using activated sludge [(102)].

3.2 Sample Collection

Water samples were collected from two effluents of Centro Hospitalar do Baixo Vouga – Aveiro, representing the wastewaters of hospitalizations and Medicine I ward and of the microbiology laboratory, and from two effluents of the WWPT of Cacia – Aveiro, which include the wastewaters of the pre-treatment and post-secondary treatment effluents, respectively.

Water samples from hospital effluents were collected at 20th November 2014 and 29th June 2015. Due to the discontinuous nature of the wastewaters from the hospital wards (Medicine I) and of microbiology laboratory, a composite sampling method was adopted and the samples were collected three times throughout the sampling day (morning, noon, and evening) and then mixed together in order to have a representative sampling of these services. Water sampling of WWTP was performed in a single collection at 27th November 2014 and 29h June 2015. Samples were collected in 1L autoclaved bottles and kept at 4 °C until DNA extraction.

The following flowchart shows a schematic representation of the samples collection (**Figure 11**).

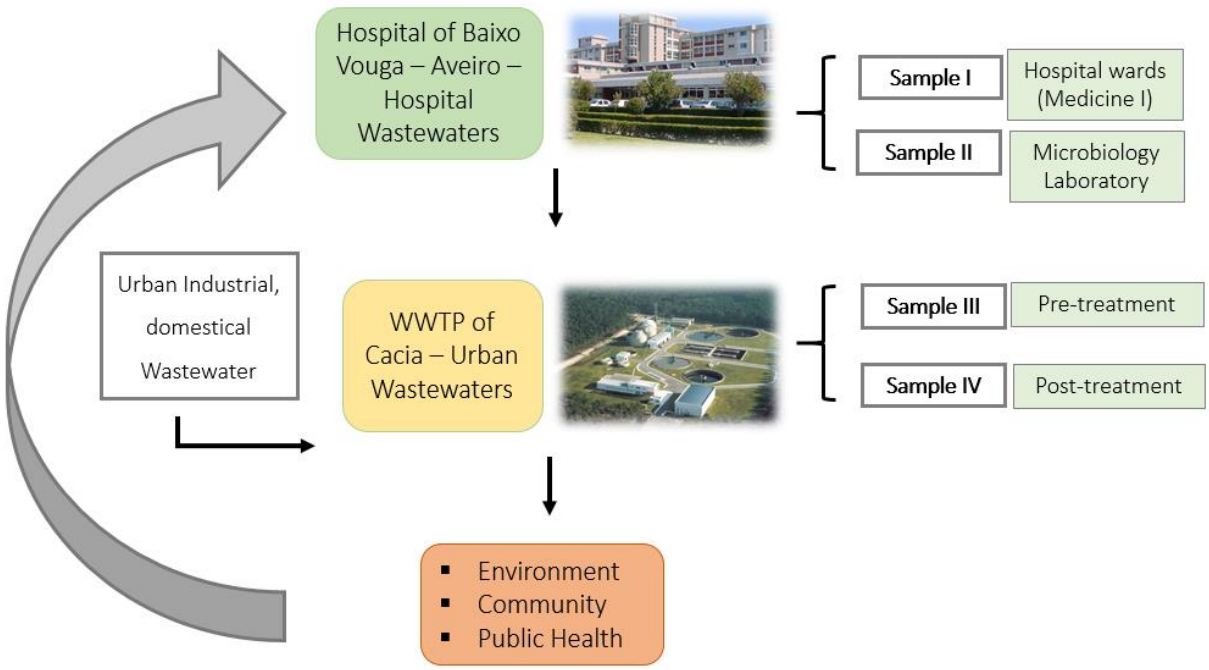


Figure 11: Flowchart with the sites and samples description.

3.3 DNA Extraction

Water samples (50 to 100mL) were pre-filtered through 5µm pore-size polycarbonate sterile filters to eliminate small eukaryotes and suspended particles and then bacterial cells were collected on 0.2 µm size filters. Cells were immediately washed from the filters using 2mL of TE buffer (10mM Tris-HCL, 1mM EDTA, pH 8.0) and suspended particles were harvested by centrifugation (16,000g for 15min). The pellet was resuspended in 100µL TE buffer containing 10mg/mL of lysozyme and incubated for 1h at 37 °C to improve cell lysis. After the described lysis steps, the extraction of DNA was performed with the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania), according to the manufacture's instruction (**Appendix 1**). Aliquots were loaded onto 0.8% agarose gels and separated by electrophoresis at 100 V for 50 min.

In order to remove all the RNA present, 5 µL of 10mg/mL DNase-free RNase A (Roche) was added, followed by an incubation at 37 °C during 1h. DNA purification was performed by Phenol-Chloroform-Isoamyl Alcohol (phenol/CIA) treatment. Deproteinization was performed by adding 1 volume of phenol/CIA, followed by centrifugation of 5 min at 14000 x g. The upper aqueous phase was transferred to a clean and sterile microtube. 1 volume of CIA was added and the upper aqueous phase was transferred to a new sterile microtube. Briefly, 1/10 volumes of a 0.5 M sodium acetate solution (pH 5.2) and 2.5 volumes of cold ethanol (-20 °C) were added to the aqueous phase obtained from the phenol/CIA extraction. The solution chilled at – 70 °C for 1h. After centrifugation for 30 min in a refrigerate centrifuge (4 °C) at maximum speed, the ethanol was removed and the pellet washed with 300 µL of 70 % ethanol. This solution was removed by centrifugation during 5 min at 13000 rpm and the pellet was dried, and resuspended in 100 µL of TE buffer. Samples were stored at -20 °C. Aliquots were loaded onto 0.8% agarose gels and separated by electrophoresis at 100 V for 50 min.

3.4 Amplification of the 16S rRNA gene

16S ribosomal RNA gene (rRNA) amplicon analysis remains the standard approach for the cultivation-independent analysis of microbial diversity and for the DNA quality of the template. Samples dilutions were performed to observe the possible presence of inhibitors. The dilutions of the samples, reduce the template concentration, which automatically result in a dilution of the PCR inhibitors.

The general bacterial primers fD1 (5'-AGAGTTTGATCCTGGCTCAG–3') and rD1 (5–AAGGAGGTGATCCAGCC–3') corresponding to positions 8–27 and 1522–1538 on *Escherichia coli* 16S rRNA gene, were used to amplify nearly full-length 16S rRNA genes [(103)]. Each reaction was performed in a final volume of 25 µL containing 50 -100 ng of purified DNA and a mix with 3 mM $MgCl_2$, 1X PCR buffer (PCR buffer without $MgCl_2$:PCR buffer with $(NH_4)_2SO_4$, 1:1), 5% DMSO, 0.2 mM dNTPs, 0.3 pmol/µL of each primer and *Taq* polymerase NZYTech (0.02U/ µL). The temperature profiles were as follows: initial denaturation (94 °C for 9 min); 30 cycles of denaturation (94 °C for 30 s), annealing (56 °C for 30 s) and extension (72 °C for 90 s); and final extension (72 °C for 10 min). The reactions were carried out in a Bio-Rad C1000TM Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). PCR products were separated by electrophoresis in a 1% agarose gel at 100 V for 50 min.

3.5 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is a fingerprinting method which provides insights into the structure and dynamics of microbial communities, being commonly used in the analysis of bacterial diversity and community structure in water samples. As a pioneer group, Muyzer and his co-workers analyzed bacterial communities of marine sediments and biofilms isolated from wastewater treatment reactors [(3,104)]. One of the biggest advantages is related with its reproducibility and ability to compare multiple samples simultaneously on the same gel [(105)].

This molecular approach allows the separation of small amplicons normally up to 400pb, which is achieved according to their different guanine and cytosine content and distribution. Before performing electrophoresis, the PCR products are obtained from environmental DNA using primers for a specific molecular marker (16S rRNA gene), with a 5'-GC clamp (30-50 nucleotides) in the forward primer, which is crucial to prevent the two DNA strands to complete dissociate into single strands. Therefore, the fingerprint pattern is created according to the melting features of the sequences along a linear denaturing gradient, which is obtained by using denaturing chemicals. In this analysis, each band corresponds to a distinct taxonomic group, resulting in a profile that can be compared. It is assumed that the variations between the intensities of bands in the DGGE gel reflect the relative abundance of different phylotypes in the bacterial communities [3,105].

DGGE was performed to analyse the following samples, I, II, III and IV. The V3 region of bacterial 16S rRNA was amplified using the primers 338F (5'-GACTCCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCCGCTGCTGG-3') with a GC clamp attached to the forward primer [104]. PCR was performed in 50 µL reaction mixtures containing 1X PCR buffer (PCR buffer without $MgCl_2$:PCR buffer with $(NH_4)_2SO_4$, 1:1), 3 mM $MgCl_2$, 5% DMSO, 200 µM each nucleotide, 0.3 pmol/µL of each primer and *Taq* polymerase NZYTech (5U/ µ). Negative controls without template DNA were run for the primer set to ensure absence of contaminating template DNA.

The temperature profile was as follows: initial denaturation (95 °C for 5 min); 30 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 30 s); and a final extension (72 °C for 30 min). The reactions were carried out in a Bio-Rad C1000TM Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA).

DGGE was performed on a DCode™ Universal Mutation Detection System (Bio-Rad, USA). Samples containing approximately equal amounts of PCR amplicons were loaded onto 8% polyacrylamide gels (37.5:1, acrylamide/bisacrylamide) using a denaturing gradient ranging from 37% to 65% (100% denaturant is 7 M electrophoresis-grade urea (Sigma-Aldrich, Germany), 40% v/v formamide (Sigma-Aldrich, Germany)). Electrophoresis was performed at 60 °C in 1x TAE buffer, at 20 V cm^{-1} for the first 15 min and then at 75V cm^{-1}

for 16h. After electrophoresis, the gels were stained for 5 min with ethidium bromide (0.5 mg L^{-1}) and then rinsed in dH_2O for 20 min. The image was acquired using a DGel DocTM XR+ (Bio-Rad Laboratories, Hercules, California, USA).

The DGGE banding patterns were analyzed with GelCompar II version 6.1 software (Applied Maths, USA). Similarities between the banding patterns were studied using the Pearson correlation coefficient based on the relative intensity of each band. Similarities were displayed graphically as a dendrogram. The clustering algorithms used to calculate the dendrograms was an unweighted pair-group method with arithmetic averages (UPGMA).

3.6 Screening for Antibiotic Resistance Genes by PCR

The PCR reactions were performed in a final volume of 25 µL containing 50 -100 ng of purified DNA and a mix with 3 mM $MgCl_2$, 1X PCR buffer (PCR buffer without $MgCl_2$:PCR buffer with $(NH_4)_2SO_4$, 1:1), 5% DMSO, 0.2 mM dNTPs, 0.3 pmol/µL of each primer and *Taq* polymerase NZYTech (0.02U/ µL). The reactions were carried out in a Bio-Rad C1000TM Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA).

bla$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{VIM}$ and *intI*1 genes were amplified with specific primers. The sets of primers used, references, annealing temperatures and predicted amplicons sizes are shown in **Table 3**. The temperature profile was as follows: initial denaturation (94 °C for 4 min); 30 cycles of denaturation (94 °C for 30 s), annealing (as indicated in **Table 3**) for 30 s, and extension (72 °C for 45 s); and a final extension (72 °C for 5 min).

PCR products were separated by electrophoresis in a 1.5% agarose gel at 100 V for 50 min.

Table 3: Primers used in this study

Target	Primer Pair	Primer Sequence (5'–3')	Annealing (°C)	Amplicon Size (bp)	Reference
bla_{CTX-M}	CTX.M_F	ACCAACGATATCGCGGTGAT	62	101	Colomer-Lluch, Jofre, & Muniesa, 2011
	CTX-M_R	ACATCGCGACGGCTTTCT			
bla_{TEM}	TEM_F	CATTTTCGTGTCGCCCTTA	59	167	Yang et al., 2012
	TEM_R	GGGCGAAAACTCTCAAGGAT			
bla_{SHV}	SHV_F	GCGAAAGCCAGCTGTCGGGC	64	304	Jiang et al., 2013
	SHV_R	GATTGGCGGCGCTGTTATCGC			
bla_{VIM}	VIM_F	TCCGACTTTACCAGATTGCC	57	159	Falcone et al., 2009
	VIM_R	CGAGAAGTGCCGCTGTGTTT			
*intI*1	IntI_F	GCCTTGATGTTACCCGAGAG	62	196	Barraud, Baclet, Denis, & Ploy, 2010
	IntI_R	GATCGGTCGAATGCGTGT			

In **Table 4** are described bacterial strains used as controls in this study, some relevant characteristics, their origin and references.

Table 4: Bacterial strains used in this study

Strain	Genes	Characteristics	References
E. coli 537	bla_{CTX-M}	*Escherichia coli* collected from patients during a control study	Unpublished
E. coli A7	bla_{SHV}, bla_{TEM}	*Escherichia coli* isolated from seawater	Alves et al., 2014
Klebsiella pneumoniae Kp1	*intI*1	*Escherichia coli* collected from an inanimate surface	Santos, Caetano, Ferreira, Ramalheira, & Mendo, 2011
Pseudomonas P1	bla_{VIM}	*Pseudomonas* collected from patients during a control study	Unpublished

3.7 Quantification of Antibiotic Resistance Gene by quantitative PCR

Quantitative PCR is a highly sensitive technique which basic goal is to distinguish and measure specific nucleic acid sequence in samples even if there is only a small quantity. The amount of DNA is measured after each cycle via fluorescent dyes that yield increasing fluorescent signal in direct proportion to the number of PCR product molecules (amplicons) generated, which is in contrast with end-point detection of amplified genes in conventional PCR, once it does not enable accurate quantification of nucleic acids [(3)].

qPCR has been used successfully in environmental samples for quantitative detection of important physiological groups of bacteria and for quantification of antibiotic resistance genes, representing an important culture-independent tool for the identification of ARGs and for the measurement of the abundance and expression of taxonomic and functional gene markers in aquatic environments [(112)].

To quantify the number of ARGs we analyzed the total DNA from wastewater and hospital effluent samples using quantitative PCR (qPCR) through absolute quantification. Absolute quantification is based in a standard curve generated from the amplification of known concentrations of the target genes (standards) in order to measure the true copy number of a particular gene [(113)].

3.7.1 Preparation of Standard Curves

For the generation of the standard curves, a plasmid construction was used. The fragments of the ARGs were amplified by conventional PCR with the specific primers for qPCR as described previously in **Table 3**.

The PCR reactions were performed in a final volume of 25 µL containing 50 -100 ng of purified DNA and a mix with 3 mM $MgCl_2$, 1X PCR buffer (PCR buffer without $MgCl_2$:PCR buffer with $(NH_4)_2SO_4$, 1:1), 5% DMSO, 0.2 mM dNTPs, 0.3 pmol/µL of each primer and

Taq polymerase NZYTech (0.02U/ µL). The temperature profile was as follows: initial denaturation (94 ºC for 4 min); 30 cycles of denaturation (94 ºC for 30 s), annealing (as indicated in **Table 3** for 30 s), and extension (72 ºC for 45 s); and a final extension (72 ºC for 5 min). PCR products were separated and confirmed by electrophoresis in a 1% agarose gel at 100 V for 50 min. Afterwards, PCR products were purified with the NZYGelpure (NZYtech) as described in **Appendix 3.**

These PCR products were ligated into the pNZY28-A plasmid vector through the NZY-A PCR Cloning Kit (NZYtech) according to the manufacturer's instructions. Ligations were carried out in a total volume of 10µL containing 1µL of pNZY28-A plasmid vector (50 ng/µL), 1µL of the fresh purified PCR product (50 ng/µL), 5 µL of NZY-A ligase buffer (10X) and 1 µL of T4 DNA Ligase (2U/µL). The mixture was incubated overnight at 4 ºC and after it, conserved at -20 ºC until further use. Approximately 10 µL of this reaction was used to transform chemically competent *E. coli* DH5α cells (**Appendix 4**). Positive clones were selected based on ampicillin resistance and white/blue selection by incubation on LB agar plates containing 100 µg/mL of ampicillin, 100 µg/mL of X-Gal and 0.5 mM of IPTG (**Appendix 4**).

Mini-preparations were used for plasmid DNA extraction from the positive clones and were performed with NZYMiniprep NZYtech kit as explained in **Appendix 5**. Plasmids harboring the inserts were linearized by digestion with the *Eco*RI endonuclease. The digestion reaction was performed in a total volume of 20 µL containing 5 µL of the resulting plasmid DNA, 1 µL of *Eco*RI (10 U/µL), 2µL of Buffer *Eco*RI (10X) and 12 µL of nuclease-free water, followed by two incubations: the first one of 37 ºC during 1h (digestion reaction) and the second one of 65 ºC during 20 min (enzyme inactivation). Each digestion was loaded on a 1% agarose gel and electrophoresis was performed at 120V during 30 min.

At the same time and in order to confirm the presence of the intended DNA fragments in the respective plasmids, a PCR was performed using the universal primers T7prom (5'-AATACGACTCACTATAGGGAGACCAC-3') and M13 Reverse (5'-CAGGAAACAGCTATGAC-3'). Each reaction was performed in a final volume of 25 µL containing 3 mM $MgCl_2$, 0.22 mM

dNTPs, 1X PCR buffer (PCR buffer without $MgCl_2$:PCR buffer with $(NH_4)_2SO_4$, 1:1), 0.3 pmol/µL of each primer. *Taq* polymerase NZYTech (0.02U/µL) and 1 µL of each intended plasmid DNA. The amplification parameters were as follows: 94 °C for 4 min, 35 cycles of 94 °C for 30 sec, 45 °C for 30 sec and 72 °C for 45 sec and a final extension step of 5 min at 72 °C. The presence of the correct amplicons was evaluated on 1.5 % agarose gel electrophoresis. These plasmid DNA containing bla_{CTX-M}, bla_{SHV}, bla_{TEM}, bla_{VIM} and *intI*1 genes were stored at -20 °C until further utilization.

The plasmid DNA concentration was determined by Qubit (Invitrogen) according to manufacturer's instructions (**Appendix 6**). Copy number of each ARGs per microliter of plasmid solution was calculated by the expression:

$$\text{Copy Number of ARG/µL} = (L \times C) / (N \times M \times 10^9)$$

According with this expression, L is Avogadro's constant (6.02×10^{23}/ mol), C is the mass concentration of plasmid in nanogram per microliter, N is the length of template containing the ARG (**Table 3)** and pNZY28-A plasmid vector (2886 bps), and M is the molecular weight of an average base pair of DNA (660 g/mol) [114].

Five point standard curves for qPCR were generated using ten-fold serial dilutions of the plasmid carrying target genes, from 10^7 to 10^2 copy numbers. Standard curves were generated using known quantities of cloned target genes. These ten-fold serial dilutions of plasmid DNA were amplified in triplicate to establish the standard curve for each qPCR assay. Each standard was generated by a correlation coefficient (R^2) of the plotted points.

3.7.2 Quantification of ARGs

qPCR reactions were conducted in 96-well plates with a final volume of 20 µL containing 10 µL of SsoFast™ EvaGreen® Supermix (Biorad, USA), 1 µl of DNA and 25 nM primers. The SoFastTM EvaGreen® Supermix was used according to manufacturer's instructions. Specific primers were designed based on the sequence of the genes to be analyzed.

Primers used in each qPCR assay and the respective reference, annealing temperatures and predicted amplicon sizes are given in **Table 3**.

The Thermal cycling and fluorescence detection were performed using the CFX96™ Real-Time System (Biorad, USA, coupled with the C1000TM Thermal Cycler (Bio-Rad). Briefly, an initial setup of 30 s at 95 ºC was followed by 40 cycles of 10 s of denaturation at 95 ºC and 15 s of annealing/extension at the temperature described in **Table 3** according to each primer. Melting curves were performed to identify the presence of primer dimers and to analyse the specificity of the reaction. The negative control was the reaction mix with nuclease-free water instead of template DNA analysis.

All negative controls resulted with no amplification or a higher threshold cycle (Ct) than the most diluted quantification standard (10^2 copy number). A sample was considered to be below the limit of detection (LOD) for a target gene if ≥2 out of 3 replicates were negative or if sample C_t values were $\geq C_t$ of negative controls. Samples above LOD were considered to be below the limit of quantification when the standard deviation (s.d.) of Ct values of methodological triplicates was >0.5 and their C_t value was higher than the Ct of the most diluted standard whose standard deviation of C_t values was ≤0.5 [115].

In order to evaluate the presence of inhibitors, each of the samples was spiked with 10^5 copies of quantification standard DNA and amplified together with the same set of non-spiked samples and control DNA. The experimental difference was compared to the true copies of target genes in the standards. The presence of inhibitors was evaluated according to Pei et al. (2006) by calculating the suppression factor [115,116]. Suppression factor was calculated according to the following formula:

$$\text{Suppression factor} = \frac{[10^5 + \text{Copy number of sample}]}{\text{Copy number of sample spiked with } 10^5 \text{ copies of standard DNA}}$$

All qPCR products were electrophoresed on 1.5% agarose gel in order to confirm the presence of a single band with the expected size. The concentrations of gene copy number were presented in gene copy number per milliliter of sample.

3.8 Statistical Analysis

Regarding to comparisons of genes abundance among the different sampling points. The statistical analysis was performed using one-way ANOVAs and Kruskal-Wallis one-way analysis of variance on ranks, whenever the data failed to meet the normality and homoscedascity assumptions. Differences were considered significant at p <0.05. All statistical analyses were performed using SigmaPlot 11.0 software.

4. Results and Discussion

4.1 DGGE analysis of bacterial community structure

DGGE analysis of 16S rRNA gene PCR products was used to investigate bacterial community structure in hospital wastewaters and in urban effluents from the WWTP of Cacia. The DGGE profiles obtained are shown in **Figure 12**.

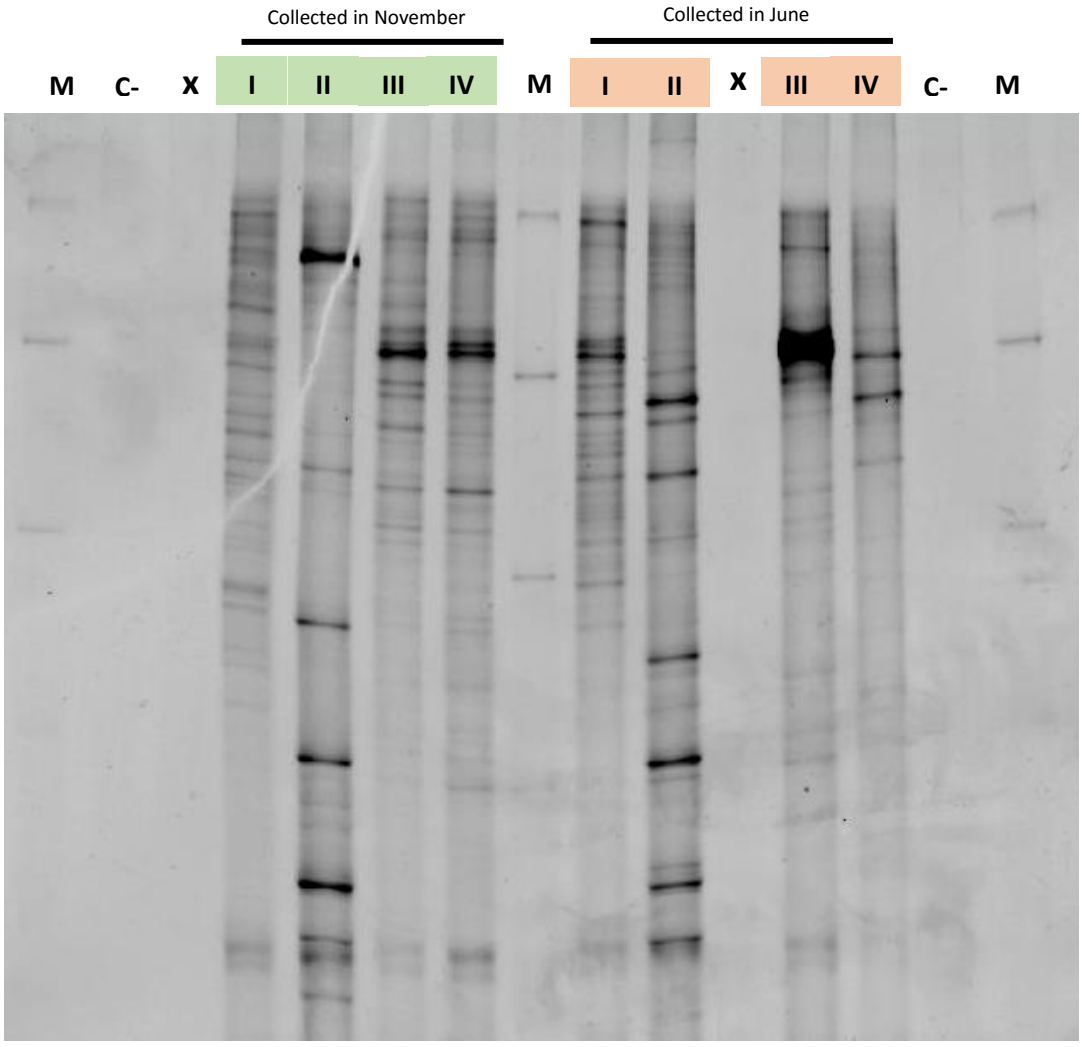


Figure 12: DGGE gel showing PCR-amplified bacterial 16S rRNA gene fragments for 8 samples (indicated on the top of each lane) collected in November 2014 and June 2015. **M:** Marker I (Wako, Japan); **I**: Hospital-medicine effluent; **II**: Hospital-microbiology laboratory; **III:** Pre-treament effluent from WWTP; **IV**: Post-treament effluent from the WWTP; **C-**: Negative control of the each amplification; **X** :lanes not considered.

Based on DGGE banding profiles (**Figure 12**), a dendrogram was constructed (**Figure 13**). To establish relationships between samples, the densitometric curves were compared by using Pearson correlation coefficient. The cluster analysis was performed to gain an overview on the relatedness of profiles representing the communities of each site and of each sampling date.

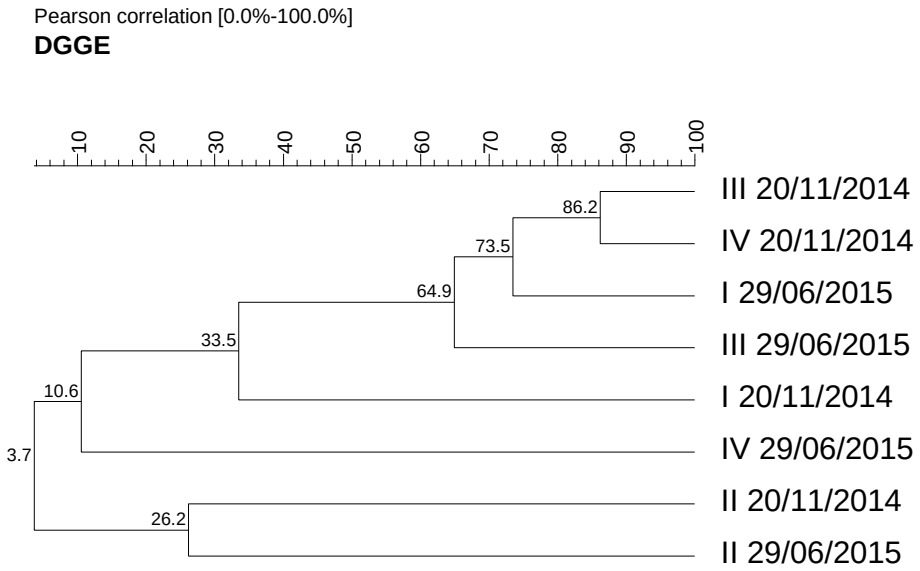


Figure 13: Cluster analysis of bacterial communities based upon DGGE profiles. Similarities were calculated using Pearson correlation.

The total number of band positions detected in this DGGE gel was 192 and the number of DGGE bands per sample varied between 16 and 29. According to the dendrogram, there is a clear separation in 2 groups: communities of the wastewaters of hospital microbiology laboratory from those of hospitalization and the urban wastewaters.

Profiles from the two sewage samples from the hospital microbiology laboratory (sample II) were located in one cluster due to their similarity (Pearson coefficient, 26.2%). These samples were collected from the same effluent in two different seasons. The temporal factor may not be more important than the spatial on the determination of this bacterial assemblage's composition. Some of the bands detected in these two effluents suggest that the populations represented by these bands may persist and colonize the laboratory

settings. Although these bacterial populations can be discharged via wastewater, these bacterial populations may represent the dominant bacteria of the microbiology laboratory.

By the dendrogram, it is visible a high similarity (86.2%) between the bacterial communities from the pre- and post-treatment effluents of the WWTP in November 2014 (samples III and IV). These samples were collected at the same day and at the dendrogram, they are located at the same cluster, sharing several bands. This result may suggest that the populations represented by these bands may persist from the effluent untreated to the final effluent of wastewater treatment plant. With this fact, it is possible to infer that there are not relevant changes in the structure of communities after treatment, leading us to believe that the treatment applied at this season may not have been effective at all or can have moderate removal efficiency.

It is important to focus in the type of treatment applied in the WWTP of Cacia – secondary treatment. Secondary treatment is a biological process to treat wastewaters by using different types of microorganisms in a controlled environment and the organic matter is consumed by these microorganisms. Generally, several aerobic biological processes can be used, which differences are related with the way oxygen is supplied to the microorganisms and with the rate at which organisms metabolize the organic matter [(31)].

According with DGGE profiles of samples III and IV collected in June 2015, it is visible that some of the dominant bacterial populations of the raw sewage were not present in the final treated effluent. This result is similar with what was obtained by Liu et al. (2007). They investigated by DGGE analysis the bacterial community structures in two WWTP with different processes and performance. The two WWTP revealed similar communities structures of raw sewage and activated sludge, but they had different populations in the effluents after treatment [(117)].

The structure of microbial communities in raw sewage are less explored then the ones on activated sludge and on the final treated effluent. Bacteria from urban sewage are

considered to derive from the soil and sanitary wastes. Sanitary wastes are known to possess enteric bacteria from human or animal excretions. Rozen and Belkin (2001) demonstrated that some bacterial populations of enteric microorganisms were difficult to detect in aeration tanks due to their reduced survival in non-host environments, showing that many enteric bacterial population may decrease in receiving waters [118]. As regard to soil bacteria, actinomycetes are the most abundant bacterial group in soils [119]. Actinomycetes are active in the organic matter decomposition in soil and can degrade agricultural and urban wastes [120]. However, when soil bacteria are mixed into wastewater, since the levels of oxygen decrease, it is expected that some of them become inactivated. So, it is reasonable that bacterial communities structures in pre- and post-treatment are different, and that the treatment applied reduces and changes the structure of these communities.

As described in the dendrogram, although sample I (hospital wards effluent) is derived from the hospital environment, it is more related with samples III and IV (urban effluents), showing a clear difference compared with sample II that also belong to the hospital environment. The microbiology laboratories (sample II) are characterized as a specific space with a particular design created based on the nature of the materials and samples handled, as well as the respective laboratory procedures. This design is essential to the safety of health professionals, hospital staff, patients, visitors and external individuals to the hospital. Restricted areas as the microbiology laboratory, comprise an invaluable and isolated part of the total health care and exams provided to patients, meaning that the access is restricted to health professionals [121,122]. On the other hand, hospitalizations placements compromise both patients and workers of the hospital, but it also include the external people that enter, visit and exit these same zones, showing involve a larger flow of internal and external individuals to the hospital. Once hospitalization settings are not an isolated place as the microbiology laboratory, it is possible that bacterial communities of hospitalizations may depend on the amount and flow individuals, reason why the phylogenetic profiles can be more related with the profiles of municipal effluents.

Another fact that corroborates this relatedness between hospitalization and urban wastewater can be supported by the absence of a wastewater treatment station for the hospital effluents. These hospital effluents are discharged into urban network without a preliminary treatment, collected to the WWTP of Cacia and co-treated with the urban wastewaters [13,27].

According to their origin of hospital wastewaters and urban wastewaters, it is expected differences in chemical and microbiological characteristics, such as some common macropollutants and major differences in the constitution and diversity of micropollutants, and then, reflecting different bacterial species composition. In this study, hospital wastewaters are co-treated with urban wastewaters at the WWTP of Cacia. In the first place, hospital effluents are discharged into urban network without a preliminary treatment. Consequently, it occurs the dilution of hospital effluents by total municipal sewage, mixing the compositions of each effluent and some components in the hospital wastewater may result in inhibition of the biomass and reduce the removal efficiency [123].

It is also supposed that hospital effluents may have generally a weak microbiological load due to the wide use of disinfectants [124]. However, the usage of these bactericides can result in negatives impacts on the biological treatments of WWTP [12,13]. Even considering, the final diluted mixture of hospital and urban effluents discharged from the WWTP after the treatment processes, there will be always possible that certain substances may create promoting effects for the biological imbalance of aquatic ecosystem and surrounding environment [27]. Some similarities between bacterial community structure of hospital effluents and municipal wastewaters, considering pre- and post-treatment effluents, can suggest the strong influence of the hospital discharges, as the efficiency of the treatment applied to the WWTP [125].

In this study, it was not possible to determine the phylogenetic affiliation of DGGE bands, in order to achieve a clear characterization of the bacterial communities present in the studied effluents. If this strategy had been applied, the bands with higher intensity, the most frequent in samples or even the different bands that distinguish DGGE profiles, could had been excised for further analysis. Once the bands were excised from the gel,

the bands could be used for re-amplification with the original set and sequenced or blotted onto nylon membranes and hybridized to molecular probes specific for different taxonomic groups [3]. To determinate the closest phylogenetic relatives, band sequences could be compared with other nucleotide sequences deposited in GenBank nucleotide data library using the BLAST software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/).

DGGE patterns can be also examined using two indexes, in order to scope various aspects of microbial diversity. The Shannon-Weaver index of diversity, *H* [126] and the equitability index, *E* [127], assuming that each band corresponds to a single species and the intensity reflects the relative abundance of species in the community. The Shannon-Weaver index takes into account the richness (the number of different species in the sample) and the dominance (higher prevalence of certain species) of certain species in the studied profile. This index will be greater the higher the richness or the more uniform are the relative intensities to these species [126]. The equitability index of Pielou (E) varies between 0 and 1. The minimum value indicates the pronounced predominance of certain species (relative intensity greater) up to a maximum which ensures the same relative abundance for all species [127]. These indexes are calculated according to the following formulas:

$$H = -\sum (n_i/N)\ \log(n_i/N),$$

$$E = H/\log\ S,$$

Where n_i represent the intensity of a particular band in a given sample and *N* is the sum of the intensities of all the bands of the same sample; *H* is the Shannon-Weaver index calculated before and *S* is the number of DDGE bands used to indicate the number of species [128].

Regarding with the studies of microbial communities, multivariate analyses have been also applied in microbial ecology. Multivariate analysis explores the relationship between dependent variables. The canonical correspondence analysis (CCA) is a multivariate

method whose aim is to explain the relationship between two sets of variables, looking for linear combinations for each of the sets of variables in order to maximize the possible correlation between groups [129,130].

There are studies that through this multivariate analysis method, they compared changings in communities structures and percentage of cultivable bacteria resistant to antibiotics in function of time, type of water, antibiotic concentration and other operating parameters [32]. In microbial ecological, is used to elucidate the relationships between biological assemblages of species and their environment [130].

With these tools, future researches may be conducted in order to study the structure of bacterial communities, diversity, evenness and richness of bacterial communities of the study.

4.2 Presence of ARGS

The ARGs conferring resistance to ß-lactam (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, and *bla*$_{VIM}$) and class 1 integron integrase (i*nt*I 1) were selected for PCR screening based on the following criteria: (i) clinically relevant genes; (ii) genes conferring resistance to frequently used β-lactam antibiotics (penicillins) and newer extended-spectrum ß-lactams (carbapenems and cephalosporins); and (iii) ARGs previously reported in mobile genetic elements [131,132].

Occurrence of β-lactamase genes was detected by PCR amplification, using primer sets targeting conserved regions of β-lactamase encoding sequences. The occurrence of integrons in the microbial populations of wastewater samples was also investigated, specifically class 1 carrying the integrase gene *int*I. For each set of primers, annealing temperatures were optimized experimentally in order to obtain specific amplification. PCR conditions were rigorously tested using DNA and in all cases the observed size of the amplicons corresponded to the expected size. Positive and negative controls were included in each assay. The PCR products were visualized on an 1.5% agarose gel.

Table 5: Screening for genes.

			bla$_{CTX-M}$	*bla*$_{SHV}$	*bla*$_{TEM}$	*bla*$_{VIM}$	*int*I 1
November 2014	Hospital	I	-	+	+	+	+
		II	-	-	+	-	+
	WWTP	III	+	+	+	+	+
		IV	+	+	+	+	+
June 2015	Hospital	I	+	+	+	+	+
		II	-	+	+	+	+
	WWTP	III	+	+	+	+	+
		IV	+	-	+	+	+

Legend: + positive result
- negative result

By conventional PCR, we obtained a qualitative analysis of the antibiotic resistance genes present in hospital and WWTP wastewater.

As summarized in **Table 5**, DNA sequences representing all antibiotic resistance genes studied were detected in all wastewater samples, revealing the presence of these resistance genes in the municipal environment as well in the hospital community.

Almost all genes were detected in all effluents. The most abundant ESBL types are represented by SHV, TEM and CTX-M, which have been described in hospital communities and in environmental samples [78,79,133]. For a long time, the most prevalent ESBLs detected were variants of SHV and TEM families, which originated by single/multiple mutations on original SHV-1 and TEM-1 penicillinase [79]. The *bla*$_{TEM}$ is one of the most frequently detected plasmid-borne antimicrobial resistance gene. However, in the last decade, *bla*$_{TEM}$ and *bla*$_{SHV}$ have become less frequent in clinics and have been replaced more recently by *bla*$_{CTX-M}$ [84,134]. In recent years, a dramatic increase in these β-lactamases has been found worldwide in different compartments [73].

In our study, *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ genes were detected. Several studies have reported the occurrence of ESBL-producing bacteria and the detection of TEM, SHV and CTX-M β-lactamases in hospital wastewaters, municipal wastewaters and WWTP effluents [135–138].

According to our results, *bla*$_{VIM}$ gene was most frequently detected in urban and hospital wastewater. VIM is one of the most relevant metallo-enzyme, which has been mostly described in clinical settings and identified in clinically pathogens as *Pseudomonas* spp., *Acinetobacter* spp. and enterobacterial clinical isolates [139,140]. *bla*$_{VIM}$ emerged mainly associated to integrons that can be embedded in transposons [141].

Marinescu and co-workers (2015) exanimated the resistance profiles of Gram-negative strains isolated from different types of wastewater and the receiving river and then estimated the contribution of these types of water to the environmental resistance reservoir. In their study, among *Enterobacteriaceae* strains analyzed, *bla*$_{VIM}$ was prevalent in *Klebsiella* sp. strains and *E. coli* strains isolated from hospital sewage effluent and urban

wastewater [142]. The presence of VIM environmental producer species may be due to nosocomial selective conditions and contamination by hospital wastewaters. However, the detection of *bla*$_{VIM}$ in different environmental species from freshwater emphasizes the role of water as a reservoir for these genes and as a vector promoting their dissemination [134,143].

The role of integrons in the capture and dissemination of ARGs is well recognized. These MGEs are frequently noticed in clinical pathogens as *Acinetobacter baumannii*, *Shigella* sp., *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *E. coli* [91,144]. Class 1 integrons are known to be widespread in clinical environment, where they usually harbor one or more gene cassettes responsible for resistance to a wide range of commonly used antibiotics [145]. Like in clinical contexts, class 1 integrons are widespread outside clinical environment and they can provide selective advantages to cope with a range of environmental pressures [131,146]. Several researchers focused on the abundance and distribution of class 1 integrons outside clinical environment have reported the presence of these integrons in compartments submitted to different degrees of anthropogenic disturbance [94,147–149].

Rosser & Young (1999) studied the incidence of integrons in environmental bacteria. They screened the presence of class 1 integrons in estuarine bacteria. Their results appear to indicate that the prevalence of class 1 integron in bacteria from natural environment is lower than what was detected in clinical isolates, however, it is likely that the proportion of bacteria with integrons may be more prevalent outside clinical settings than previously estimated, since less than 1% of the bacteria in environmental samples are believed to be cultivable under laboratory conditions [149].

In our study, we screened the occurrence of class 1 integrons in total DNA from wastewater samples, which was positive for both effluents, clinical settings and municipal sewage. Several studies had reported the occurrence of class 1 integrons in bacterial isolates obtained from hospital surfaces and wastewaters, livestock, fish farm, estuarine environments and other environmental sources, suggesting that integrons may be ubiquitous in nature [91,111,146,149]. A future approach that could be performed is related

with the confirmation if the class 1 integrons quantified carry the resistance genes discussed.

Some amplicons were not detectable in some samples from both municipal and clinical effluents. Conventional PCR is known as an approach that measures PCR amplification as it occur and in this study, we applied 30 cycles of temperature. However, these genes were subsequently detected in our analysis by qPCR. Since, qPCR measures the amount of accumulated PCR product at the end of the PCR cycles (normally 40 cycles), it was possible to detect the presence of these genes in lower quantities [150]. However, these results will be further explained and discussed.

Hospital effluents and other wastewaters from clinical settings may represent a risk to public health due to the presence of pathogens, chemicals and drugs that are not metabolized by patients [12,151]. Hospital wastewaters analyzed in this study are directly discharged without pre-treatment to the urban sewage network, reaching the WTTP as urban sewage. WWTP are characterized by selective pressures for resistance to many toxic compounds, high organic content and high bacterial diversity providing a bond for mobile elements to mix between pathogens and environmental bacteria. Although hospital wastewaters have different origin and composition of urban effluents, once mixed, it become difficult to differentiate the resistance determinants between them by conventional PCR, since the WWTP collects the effluents from eight municipalities.

Although many ESBL-encoding genes and mobile genetic structures have been described in the literature, this study did not carried out with the specific identification of β-lactamase genetic determinants and integron gene cassettes by sequencing. As a future approach, it could be performed the purification of PCR product followed by their nucleotide sequencing and the comparison with other sequences deposited and available in the GenBank database using the basic local alignment search tool (Blast and ClustalW computer programs) [152].

4.3 Absolute quantification by qPCR of ARGs

4.3.1 Standard curves and amplification efficiencies

From the slope of each standard curve, the reaction efficiency (*E*) was calculated according to the equation:

$$E = 10^{(-1/\text{slope})} - 1$$

The efficiency should be 100% (slope = -3.32), but this value is difficult to achieve. However, efficiency values between 90 and 110% are acceptable. The correlation coefficient (R^2) of the curve should be ≈ 1 and the y-intercept value provides information about the theoretical limit of detection of the reaction (C_t correspondent to the minimum copy number that gives rise to statistically significant amplification) [153].

According to the standard curves, the C_t value of unknown samples was used to calculate the number of corresponding gene copies. Each reaction was run in triplicate. High R^2 values (average 0.99844) and high values efficiencies (from 91.50 to 101.31%) obtained from the standard curves demonstrated the linearity and sensitivity of each qPCR assay. The efficiency, correlation coefficient, slope and y-intercept values are present in **Table 6**.

Table 6: Efficiency, correlation coefficient, slope and y-intercept

Target gene	Efficiency (%)	R^2	Slope	y-intercept
bla$_{CTX-M}$	98.64	0.9996	-3.355	39.344
bla$_{TEM}$	101.31	0.9983	-3.291	39.847
bla$_{SHV}$	91.50	0.9996	-3.544	38.992
bla$_{VIM}$	99.42	0.9997	-3.336	40.027
intI1	101.69	0.995	-3.282	43.912

4.3.2 Evaluation of PCR inhibitors

The presence of inhibitors was evaluated as described by Pei et al. (2006) through the calculation of the suppression factor. Each of the samples was spiked with 10^5 copies of quantification standard DNA and amplified together with the same set of non-spiked samples and control DNA.

Table 7: Suppression factors calculated for a subset of spiked samples in qPCR assay of each ARGs

	bla$_{CTX-M}$	*bla*$_{TEM}$	*bla*$_{SHV}$	*bla*$_{VIM}$	*intI*1
Hospitalizations	1.51	1.13	1.50	7.29	0.17
Microbiology Laboratory	2.06	1.05	1.03	0.69	8.57
Pre-treatment	2.23	1.33	1.54	3.36	0.50
Post-treatment	0.98	1.44	1.70	0.84	7.84

Suppression factors were calculated for each sample according to the gene under study. As described in **Table 7**, suppression factors demonstrate variance between themselves. These values do not become clear and conclusive enough to evaluate the presence or absence of inhibitors. However, comparing the C_t values and the amplification curves of samples, of standard 10^5 copies and of the samples spiked with 10^5 of standard, it was visible that samples spiked with 10^5 of standard amplified earlier than the normal samples and closer from the standard 10^5 copies, indicating that inhibition was negligible.

4.3.3 Abundance of resistance genes in hospital and urban effluents

After analyzing and comparing the screening of the genes in both samples collected in November 2014 and June 2015, quantification of resistance genes was only performed in the samples collected in June 2015 due to the little volume available of the samples from November 2014. The five genes were selected for quantification by qPCR and gene copy

numbers were calculated per milliliter of sample in order to compare abundances in the different effluents (**Table 8**).

Table 8: Abundance of genes (copy number gene/mL sample)

		Hospital Wards	Microbiology Laboratory	Pre-treatment	Post-Treatment	Total abundance for gene
***bla*$_{CTX-M}$**	Average	3.365×10^{4}	2.775×10^{4}	3.526×10^{4}	6.303×10^{4}	1.5972×10^{5}
	s.d	7.648×10^{2}	2.946×10^{3}	2.056×10^{3}	7.042×10^{3}	
***bla*$_{TEM}$**	Average	1.982×10^{6}	5.035×10^{5}	1.028×10^{6}	7.093×10^{4}	4.2859×10^{6}
	s.d	1.482×10^{6}	5.165×10^{5}	3.068×10^{6}	2.002×10^{5}	
***bla*$_{SHV}$**	Average	6.453×10^{5}	5.392×10^{4}	4.592×10^{5}	6.196×10^{4}	1.2205×10^{6}
	s.d	6.186×10^{4}	1.003×10^{3}	1.424×10^{4}	7.743×10^{3}	
***bla*$_{VIM}$**	Average	2.879×10^{8}	6.668×10^{5}	6.372×10^{6}	6.326×10^{6}	3.0127×10^{8}
	s.d	$1.630E\times10^{7}$	4.010×10^{4}	1.529×10^{5}	1.331×10^{5}	
***intI*1**	Average	3.002×10^{7}	6.681×10^{6}	1.538×10^{7}	2.403×10^{6}	5.4490×10
	s.d	2.390×10^{5}	1.121×10^{7}	4.508×10^{5}	1.682×10^{4}	
Total abundance for effluent		3.2059×10^{8}	7.9331×10^{6}	2.3277×10^{7}	9.6275×10^{6}	

Regarding to the total abundance of the genes for effluent (sum of abundance all genes in each effluents), hospital wards effluent was the highest, about 3.2059×10^{8} copies/mL sample, followed by pre-treatment effluent with an abundance of 2.3277×10^{8} copies/mL sample. Microbiology laboratory effluent was the lowest in total abundance (**Table 8**, **Figure 14**). However, these results were not statistically significant (Kruskal-Wallis, p = 0.658).

The total abundance for gene (sum of abundance of a gene in all effluents) varied greatly (**Table 8**, **Figure 15**). *bla*$_{VIM}$ abundance was the highest, about 3.0127×10^{8} copies/mL sample, followed by *intI*1 with a total abundance of 5.4490×10^{7} copies/mL sample. *bla*$_{CTX-M}$ abundance was the lowest (**Table 8**, **Figure 15**). There was a statistically significant difference (Kruskal-Wallis, p = 0,003).

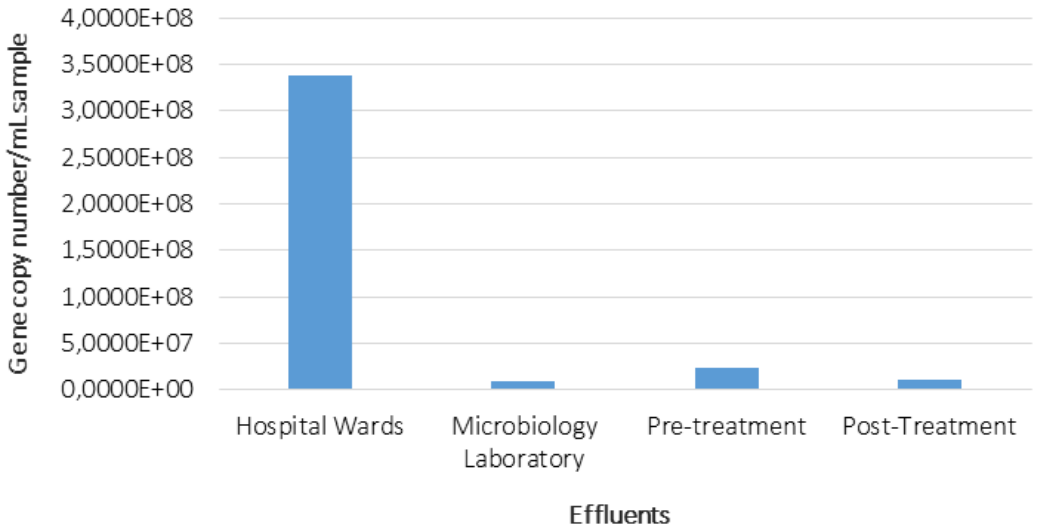


Figure 14: Abundance for effluent

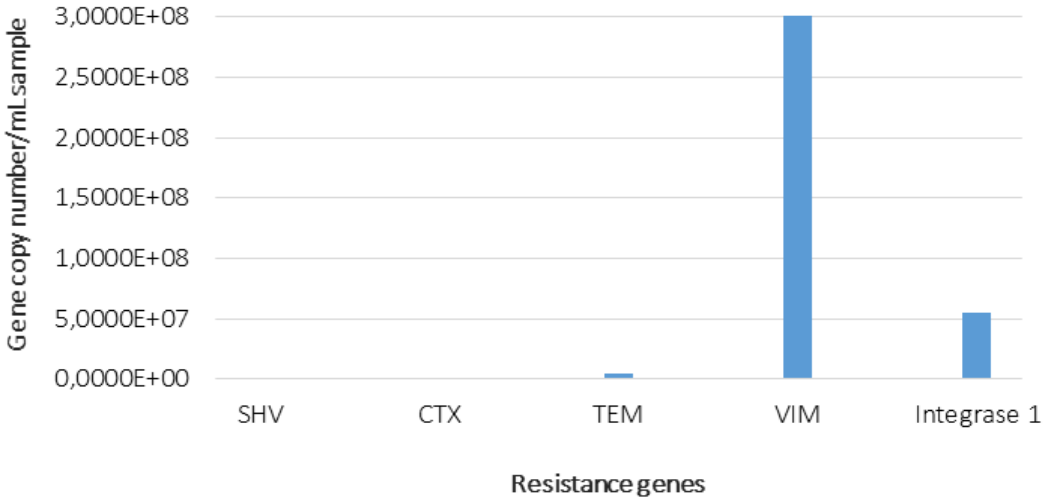


Figure 15: Total abundance for gene

In this study, it is also possible to differentiate the wastewaters between hospital (hospital wards and microbiology laboratory) and urban/municipal (pre- and post-treatment) sources. As described in results and even without statistical significance (Kruskal-Wallis, p = 0.880), hospital effluents revealed a higher number of copies of the genes compared with urban effluents (**Table 8**, **Figure 16**). Although the WWTP of Cacia

receives effluents from eight municipalities, including other hospitals and domestical/urban wastes, probably untreated hospital discharges are a potential sources of antibiotic resistant bacteria and resistance genes reaching the WWTP. Hospital wastewaters are supposed to contain higher loads of resistant organisms, acting as suppliers of resistance into the environment [34,154,155]. Conventional WWTP are normally not designed to treat hospital wastewaters and to remove associated contaminants [156]. In some European countries, different WWTPs have already been created, requiring a specific management and treatment of hospital effluents [157].

With regard to the effluents from WWTP of Cacia, there was no statistically significant difference (One Way ANOVA, p = 0.411). However, the quantitative analysis demonstrated that the abundance of all genes decreased as a result of wastewater treatment (**Figure 16**). This result suggest that treatment applied of the WWTP may have a moderate removal efficiency and it also indicates the persistence of these genes in the natural water, meaning that WWTP discharge can support their spread into aquatic environments [107,158].

The most abundant ESBL types are represented by $bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM}, being widely encountered in clinical therapy [79]. $bla_{\text{CTX-M}}$ was less abundant in all sites, followed by bla_{SHV} and bla_{TEM}, indicating that may not be the dominant β-lactamase resistance genes in these places (One Way ANOVA, p = 0,016) (**Figure 16**).

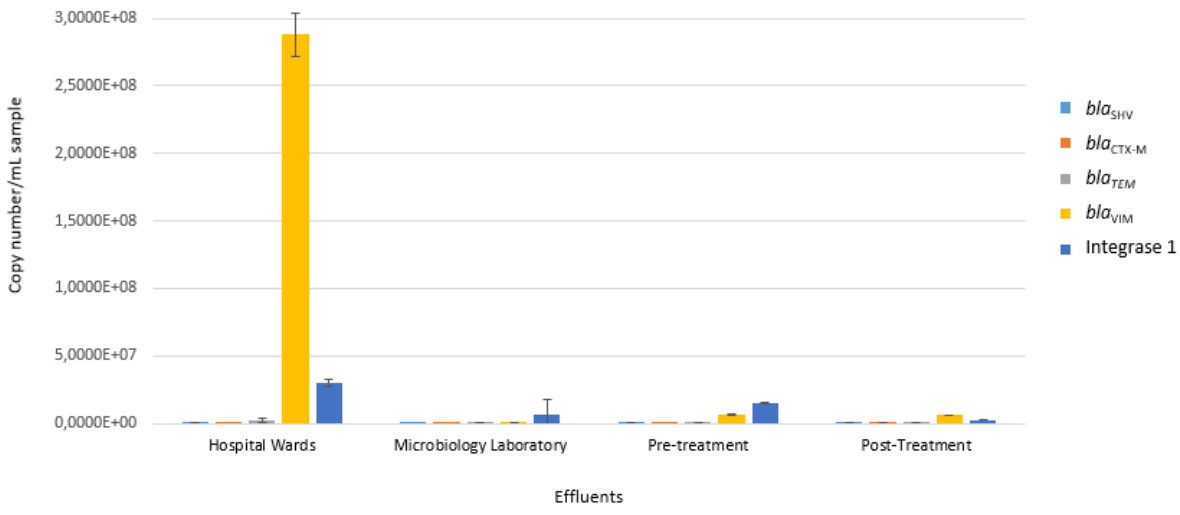


Figure 16: Abundance of genes per sample

bla$_{CTX-M}$ has been reported in the last decade as the most prevalent extended β-lactamase [73]. However, our results agree with a previous study about the occurrence and abundance of β-lactam resistance genes in wastewaters in which *bla*$_{CTX-M}$ was not even detected [107].

Plasmid-mediated bla$_{TEM}$ and ***bla*$_{SHV}$** have been found in different settings worldwide [79]. Li and co-workers (2009) analyzed the antibiotic-resistance profile in environmental bacteria isolated from penicillin production wastewater treatment plant and the receiving river in China. They figured out that *bla*$_{TEM}$ and *bla*$_{CTX-M}$ were detectable in the biological treatment effluent of penicillin-producing wastewater [159]. Yang et al (2012) demonstrated clearly the presence of TEM and other β-lactam resistance gene in activated sludge of different WWTPs in East Asia and North America [107].

It should be also stated that many of these studies quantified the *bla* genes in river water under the influence of wastewater discharges, but not directly in the treated effluent from WWTP [158,160,161]. Another report related with antibiotic resistance was performed in source water and drinking water treatment plant in Ohio and Michigan, where it the water was tested before and after treatment. Although the bacterial concentration was lower during the treatment process, the prevalence of resistance to amoxicillin, rifampin, and chloramphenicol increased, showing that antibiotic resistance persists during the water treatment. They detected *bla*$_{TEM}$ and *bla*$_{SHV}$ in almost all samples, representing an evidence that these genes are commonly distributed through water systems [162].

As regards the results of the abundance of *bla*$_{VIM}$ gene, there were statistically significant differences (Kruskal Wallis*, p* = 0.025). It is undoubtedly visible a huge abundance of 2.8791×10^{8} in wastewaters of hospitalization settings. In microbiology laboratory effluent, the *bla*$_{VIM}$ was the least abundant. The urban effluents (pre-and post-treatment) had a moderate abundance compared to the hospital effluents (**Table 8**).

MBL are known to be encoded either by genes that are part of the chromosomal framework in some bacterial species (resident MBL) or by genes acquired by HGT

(acquired MBL) [86,163]. The presence of *bla*$_{VIM}$ has been described as an acquired MBL, being mostly detected in strains of Enterobacteriaceae, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and other Gram-negative non- fermenters [86]. Several studies reported the occurrence of these acquired MBL–producing bacteria in hospital environments, revealing the occurrence of clonal spread and nosocomial outbreaks [140,164–168]. Thus, once these hospitals represent reservoirs of MBL-producing pathogens, it can be expected the high abundance of these gene in these settings and consequently, in their wastewaters.

The *bla*$_{VIM}$ gene has been mostly found in clinical settings and its role of in nonclinical habitats for bacteria that carry this gene has been less explored and probably this gene is less abundant in the environment [143]. Besides, it should be also taken into account the dilution of the hospital wastewater when it reaches the municipal sewage and when the municipal sewage mixes with all effluents from other cities collected at the WWTP [169]. These facts corroborate our results, once the *bla*$_{VIM}$ abundance was lower.

Regarding *intI*1 abundance, it was higher in hospitalization wastewaters than in microbiology effluent. According to urban effluents, *intI*1 abundance was higher in pre-treatment effluent than in the post-treatment (**Figure 16**). There were no statistically significant differences (Kruskal-Wallis, p = 0.055). Class 1 integron are found in diverse locations, even in different plasmids and transposons, being described as ubiquitous in nature. They are described in pathogens isolated from clinical settings and pathogens and commensals isolated from livestock [170,171].

The differences between hospital and urban wastewaters can be explained by the dilution factor of the effluents, as mentioned before. At the same time, the abundance reduction of *intI*1 in the outside of the WWTP reveals that the biological treatment by activated sludge reduce in fact the abundance integrons. These results agree with previous studies in which it was demonstrated by qPCR that activated sludge process reduce the abundance of integrons [172]. Anthropogenic activities can lead to the release of abundant integrons and antibiotic-resistance gene cassettes, but in this study we cannot conclude no specific impact of hospital activities on the receiving environment. A previous study

investigated the role of hospital and urban effluents in resistance spread using i*ntI*1 gene as an integron marker, concluding that the WWTP with biological treatment could reduce the diversity of gene cassette arrays in the pre-treatment effluent [99].

Wastewater treatment is not designed to remove or eliminate DNA contents and to decrease the abundance of resistance genes [100,173]. The presence of low concentrations of antibiotics in WWTPs has been documented [174,175]. The abundance of some genes may range during the treatment process as a consequence of selection driven by antibiotics and other antimicrobials that are inefficiently removed during the treatment [173,176]. Even during the treatment, there are environmental conditions of activated sludge that promote the HGT between hosts due to the nutritional richness and high bacterial density and diversity. Bacteria carrying MGEs associated with resistance genes or that acquire resistance determinants by HGT, can sometimes increase the abundance during processes of water treatment [145].

In this study, it was only quantified the abundance of the resistance genes and the copy number of each gene was calculated per mL of sample. However, it 16S rRNA gene quantification was performed, it could be possible to use the number of copies of 16S rRNA as a proxy for bacterial count to normalize the levels of β-lactamase encoding material depending on the bacterial count.

The quantification of 16S rRNA genes allows to estimate the bacterial load per sample. With the view to avoid inconsistence among qPCR assays, including suboptimal efficiency, the 16S rRNA gene-normalized values could be used. The copy number of resistance genes and 16S rRNA gene for each sample could be calculated by plotting against the respective standard curve. The former would be divided by the later in order to order to normalize for any differences in input DNA concentrations between samples. Most of the studies concerning the occurrence and abundance of antibiotic resistance genes, report gene copy numbers normalized against mass (nanogram) of the extracted DNA samples, against the volume (milliliter) of sewage or sludge samples or the copy number of total 16S rRNA [107,161,176,177].

5. Conclusions and Future Perspectives

Hospital settings and health care facilities, where antibiotic use is more frequent and intensive, are regarded as important reservoirs of antibiotic resistance and nosocomial pathogens. In addition, municipal sewage receives the bacteria previously exposed to antibiotics from private households, industries and hospitals, being recognized as receptors for antibacterial substances and antibiotic resistant bacteria. The microbial abundance, nutrients concentrations and selective pressures of antibiotics and other pollutants promotes the development of resistance in these environments and to HGT process.

In this study, microbial communities from hospital wards and pre- and post-treatment effluents are phylogenetically related. The hospital wastewaters had the highest total abundance of the genes than the urban effluents. Of all genes bla_{VIM} had a notable abundance due its high abundance in hospitalizations wastewater, followed by *intI*1. bla_{CTX-M}, bla_{TEM}, bla_{SHV} had the lowest abundances. Regarding with the biological treatment applied in the WWTP of Cacia, once the abundance of the genes was lower after the treatment, it is predictable that the treatment can be moderately effective. Our results show that hospital settings tend to be a complex environment due to the high microbial diversity of commensal and nosocomial pathogens, the selective pressures created by antimicrobial use and the events of lateral gene transfer. The urban sewage network and the WWTP-Cacia represent a receptor of antimicrobial residues and antibiotic-resistant bacteria.

Herein, it was not possible to perform the sequencing and identification of DGGE fragments. However, a following study could include this in the future and it would become clear to know exactly the bacterial populations that exist in hospital wastewaters and WWTP effluents. Additional approaches are needed to identify and quantify the most

phylogenetic groups, as quantitative slot blot hybridization or quantitative real time polymerase chain reaction [178].

Further investigations are also needed to complete evaluate the potential resistome profile and role of Aveiro Hospital wastewaters and WWTP-Cacia effluents. The search should be extended to genetic determinants conferring resistance to other antibiotic groups, as well the possible links between MGEs carrying these genetic determinants. Culturing techniques could be applied to assess the diversity of cultivable bacteria and to determinate both resistance phenotypes and genetic content in resistance determinants. Culture-independent methodologies based on RNA analysis would be useful to estimate the expression of antimicrobial resistance genes in the environment.

6. Bibliography

1. Whitman WB, Coleman DC, Wiebe WJ. Prokaryotes: the unseen majority. Proc Natl Acad Sci. 1998;95:6578–83.
2. Singh BK, Campbell CD, Sorenson SJ, Zhou J. Soil genomics. Nat Rev Microbiol. 2009;7(10):756–7.
3. Rastogi G, Sani RK. Molecular techniques to assess microbial community structure, function, and dynamics in the environment. Microbes Microb Technol Agric Environ Appl. 2011;(2):29–57.
4. Irwin J a, Baird AW. Extremophiles and their application to veterinary medicine. Ir Vet J. 2004;57(6):348–54.
5. Torsvik V, Ovreas L, Thingstad TF. Prokaryotic diversity--magnitude, dynamics, and controlling factors. Environ Microbiol. 2002;296(5570):1064–6.
6. Deming JW. Psychrophiles and polar regions. Curr Opin Microbiol. 2002;5:301–9.
7. Doney SC, Abbott MR, Cullen JJ, Karl DM, Rothstein L. From genes to ecosystems: the ocean's new frontier. Front Ecol Environ. 2004;2(9):457–68.
8. Vaz-Moreira I, Nunes OC, Manaia CM. Bacterial diversity and antibiotic resistance in water habitats: searching the links with the human microbiome. FEMS Microbiol Rev. 2014;38(4):761–78.
9. Boyce JM. Environmental contamination makes an important contribution to hospital infection. J Hosp Infect. 2007;65(S2):50–4.
10. Hosein IK, Hill DW, Jenkins LE, Magee JT. Clinical significance of the emergence of bacterial resistance in the hospital environment. Soc Appl Microbiol. 2002;92(31):90S – 97S.
11. Donskey CJ. Does improving surface cleaning and disinfection reduce health care-associated infections? Am J Infect Control. Elsevier Inc; 2013;41(5):S12–9.
12. Emmanuel E, Perrodin Y, Keck G, Blanchard J-M, Vermande P. Ecotoxicological risk assessment of hospital wastewater: a proposed framework for raw effluents discharging into urban sewer network. J Hazard Mater. 2005 Jan 14;A117:1–11.
13. Verlicchi P, Galletti A, Petrovic M, Barceló D. Hospital effluents as a source of emerging pollutants: An overview of micropollutants and sustainable treatment options. J Hydrol. 2010;389(3-4):416–28.
14. Bouki C, Venieri D, Diamadopoulos E. Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: a review. Ecotoxicol Environ Saf. 2013;91:1–9.

15. Nikaido H. Multidrug resistance in bacteria. Annu Rev Biochem. 2009;78:119–46.

16. Dzidic S, Suskovic J, Kos B. Antibiotic resistance mechanisms in bacteria: biochemical and genetic aspects. Food Technol Biotechnol. 2008;46(1):11–21.

17. Dancer SJ. The role of environmental cleaning in the control of hospital-acquired infection. J Hosp Infect. Elsevier Ltd; 2009;73(4):378–85.

18. Weber DJ, Rutala W a, Miller MB, Huslage K, Sickbert-Bennett E. Role of hospital surfaces in the transmission of emerging health care-associated pathogens: norovirus, *Clostridium difficile*, and *Acinetobacter* species. Am J Infect Control. 2010;38(5 Suppl 1):S25–33.

19. Rose M, Landman D, Quale J. Are community environmental surfaces near hospitals reservoirs for gram-negative nosocomial pathogens? Am J Infect Control. 2014;42(4):346–8.

20. Cantón R, Horcajada JP, Oliver A, Garbajosa PR, Vila J. Inappropriate use of antibiotics in hospitals: The complex relationship between antibiotic use and antimicrobial resistance. Enferm Infecc Microbiol Clin. 2013;31, Supple:3–11.

21. Jayaraman R. Antibiotic resistance : an overview of mechanisms and a paradigm shift. Curr Sci. 2009;96(11):1475–84.

22. Magiorakos A, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012;18(3):268–81.

23. Mulvey MR, Simor AE. Antimicrobial resistance in hospitals: How concerned should we be? Can Med Assoc J. 2009;180(4):408–15.

24. Zhang X-X, Zhang T, Fang HHP. Antibiotic resistance genes in water environment. Appl Microbiol Biotechnol. 2009;82(3):397–414.

25. Pauwels B, Verstraete W. The treatment of hospital wastewater: an appraisal. J Water Health. 2006;4(4):405–16.

26. Ort C, Lawrence MG, Reungoat J, Eaglesham G, Carter S, Keller J. Determining the fraction of pharmaceutical residues in wastewater originating from a hospital. Water Res. 2010;44(2):605–15.

27. Gautam AK, Kumar S, Sabumon PC. Preliminary study of physico-chemical treatment options for hospital wastewater. J Environ Manage. 2007;83(3):298–306.

28. Szewzyk U, Szewzyk R, Manz W, Schleifer K-H. Microbiological safety of drinking water. Annu Rev Microbiol. 2000;54:81–127.

29. Leclerc H, Schwartzbrod L, Dei-Cas E. Microbial agents associated with waterborne diseases. Crit Rev Microbiol. 2002;28(4):371–409.

30. Taylor NGH, Verner-Jeffreys DW, Baker-Austin C. Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? Trends Ecol Evol. 2011;26(6):278–84.

31. Sonune A, Ghate R. Developments in wastewater treatment methods. Desalination. 2004;167(1):55–63.

32. Novo A, André S, Viana P, Nunes OC, Manaia CM. Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. Water Res. 2013;47(5):1875–87.

33. Rizzo L, Michael I, McArdell CS, Manaia CM, Merlin C, Schwartz T, et al. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review. Water Res. 2013;47(3):957–95.

34. Guardabassi L, Lo Fo Wong DM., Dalsgaard A. The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria. Water Res. 2002;36(8):1955–64.

35. Hong P-Y, Al-Jassim N, Ansari M, Mackie R. Environmental and public health implications of water reuse: antibiotics, antibiotic resistant bacteria, and antibiotic resistance genes. Antibiotics. 2013;2(3):367–99.

36. Kohanski MA, Dwyer DJ, Collins JJ. How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol. 2010;8:423–35.

37. Clewell DB. Antibiotic resistance plasmids in bacteria. Encicl Life Sci. 2005;1–6.

38. Tenover FC. Mechanisms of antimicrobial resistance in bacteria. Am J Med. 2006;119(6A):S3–10.

39. Clardy J, Fischbach M a., Currie CR. The natural history of antibiotics. Curr Biol. 2009;19(11):1–8.

40. Kardos N, Demain AL. Penicillin: The medicine with the greatest impact on therapeutic outcomes. Appl Microbiol Biotechnol. 2011;92(4):677–87.

41. Suárez C, Gudiol F. Antibióticos betalactámicos. Enferm Infecc Microbiol Clin. 2009;27(2):116–29.

42. Samaha-Kfoury JN, Araj GF. Recent developments in β-lactamases and extended spectrum β-lactamases. Br Med J. 2003;327(7425):1209–13.

43. Van Hoek AH a M, Mevius D, Guerra B, Mullany P, Roberts AP, Aarts HJM. Acquired antibiotic resistance genes: An overview. Front Microbiol. 2011;2(203):1–27.

44. Barry PM, Klausner JD. The use of cephalosporins for gonorrhea: The impending problem of resistance. Expert Opin Pharmacother. 2010;10(4):555–77.

45. Rafii F, Sutherland JB, Cerniglia CE. Effects of treatment with antimicrobial agents on the human colonic microflora. Ther Clin Risk Manag. 2008;4(6):1343–57.

46. Singh SB, Young K, Miesel L. Screening strategies for discovery of antibacterial natural products. Expert Rev Anti Infect Ther. 2011;9(8):589–613.

47. Typas A, Banzhaf M, Gross C a., Vollmer W. From the regulation of peptidoglycan synthesis to bacterial growth and morphology. Nat Rev Microbiol. 2011;10(2):123–36.

48. Macheboeuf P, Contreras-Martel C, Job V, Dideberg O, Dessen A. Penicillin binding proteins: Key players in bacterial cell cycle and drug resistance processes. FEMS Microbiol Rev. 2006;30(5):673–91.

49. Palzkill T. Metallo-β-lactamase structure and function. Ann N Y Acad Sci. 2013;1277:91–104.

50. Vollmer W, Blanot D, De Pedro M a. Peptidoglycan structure and architecture. FEMS Microbiol Rev. 2008;32(2):149–67.

51. Sauvage E, Kerff F, Terrak M, Ayala J a., Charlier P. The penicillin-binding proteins: Structure and role in peptidoglycan biosynthesis. FEMS Microbiol Rev. 2008;32(2):234–58.

52. Zapun A, Contreras-Martel C, Vernet T. Penicillin-binding proteins and β-lactam resistance. FEMS Microbiol Rev. 2008;32(2):361–85.

53. Lewis K. Platforms for antibiotic discovery. Nat Rev Drug Discov. 2013;12(5):371–87.

54. Essack SY. The development of β-lactam antibiotics in response to the evolution of β-lactamases. Pharm Res. 2001;18(10):1391–9.

55. Rice LB. Mechanisms of resistance and clinical relevance of resistance to β-lactams, glycopeptides, and fluoroquinolones. Mayo Clin Proc. 2012;87(2):198–208.

56. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev. 2003;67(4):593–656.

57. Pagès J-M, James CE, Winterhalter M. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nat Rev Microbiol. 2008;6(12):893–903.

58. Livermore DM. Of Pseudomonas, porins, pumps and carbapenems. J Antimicrob Chemother. 2001;47(3):247–50.

59. Hancock REW, Brinkman FSL. Function of *Pseudomonas* porins in uptake and efflux. Annu Rev Microbiol Microbiol. 2002;56:17–38.

60. Tang SS, Apisarnthanarak A, Hsu LY. Mechanisms of β-lactam antimicrobial resistance and epidemiology of major community- and healthcare-associated multidrug-resistant bacteria. Adv Drug Deliv Rev. 2014;78:3–13.

61. Fernández L, Hancock REW. Adaptive and mutational resistance: Role of porins and efflux pumps in drug resistance. Clin Microbiol Rev. 2012;25(4):661–81.

62. Webber M a., Piddock LJ V. The importance of efflux pumps in bacterial antibiotic resistance. J Antimicrob Chemother. 2003;51(1):9–11.

63. Li XZ, Li XZ, Nikaido H, Nikaido H. Efflux-mediated drug resistance in bacteria. Drugs. 2004;64(2):159–204.

64. Mahamoud A, Chevalier J, Alibert-Franco S, Kern W V., Pagès JM. Antibiotic efflux pumps in Gram-negative bacteria: The inhibitor response strategy. J Antimicrob

Chemother. 2007;59:1223–9.

65. Wilke MS, Lovering AL, Strynadka NCJ. β-Lactam antibiotic resistance: A current structural perspective. Curr Opin Microbiol. 2005;8(5):525–33.

66. Dhillon RH-P, Clark J. ESBLs: A Clear and Present Danger? Crit Care Res Pract. 2012;1–11.

67. Bush K, Jacoby G a. Updated functional classification of β-lactamases. Antimicrob Agents Chemother. 2010;54(3):969–76.

68. Bush K. Proliferation and significance of clinically relevant β-lactamases. Ann N Y Acad Sci. 2013;1277(1):84–90.

69. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother. 1995;39(6):1211–33.

70. Summers AO. Genetic linkage and horizontal gene transfer, the roots of the antibiotic multi-resistance problem. Anim Biotechnol. 2006;17(2):125–35.

71. Alekshun MN, Levy SB. Molecular mechanisms of antibacterial multidrug resistance. Cell. 2007;128(6):1037–50.

72. Gillings MR, Xuejun D, Hardwick S a, Holley MP, Stokes HW. Gene cassettes encoding resistance to quaternary ammonium compounds: a role in the origin of clinical class 1 integrons? ISME J. 2009;3(2):209–15.

73. Cantón R, Coque TM. The CTX-M β-lactamase pandemic. Curr Opin Microbiol. 2006;9(5):466–75.

74. Giske CG, Sundsfjord AS, Kahlmeter G, Woodford N, Nordmann P, Paterson DL, et al. Redefining extended-spectrum β-lactamases: balancing science and clinical need. J Antimicrob Chemother. 2009;63(1):1–4.

75. Paterson DL. Resistance in gram-negative bacteria: Enterobacteriaceae. Am J Infect Control. 2006;34(5):S20–8.

76. Mark E. Rupp, Fey PD. Extended spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*: considerations for diagnosis, prevention and drug treatment. Drugs. 2003;63(4):353–65.

77. Falagas ME, Karageorgopoulos DE. Extended-spectrum β-lactamase-producing organisms. J Hosp Infect. Elsevier Ltd; 2009;73(4):345–54.

78. Pitout JDD. Infections with Extended-Spectrum β-Lactamase-Producing Enterobacteriaceae. Drugs. 2010;70(3):313–33.

79. Paterson DL, Bonomo RA. Extended-Spectrum β-Lactamases: a Clinical Update. Clin Microbiol Rev. 2005;18(4):657–86.

80. Bradford P. Extended spectrum betalactamase in the 21 century: characterization, epidemiology, and detection of this important resistant threat. Clin Microbiol Rev. 2001;14(4):933–51.

81. Naas T, Cuzon G, Truong H, Bernabeu S, Nordmann P. Evaluation of a DNA microarray, the check-points ESBL/KPC array, for rapid detection of TEM, SHV, and CTX-M extended-spectrum β-lactamases and KPC carbapenemases. Antimicrob Agents Chemother. 2010;54(8):3086–92.

82. Shaikh S, Fatima J, Shakil S, Rizvi SMD, Kamal MA. Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. Saudi J Biol Sci. 2015;22(1):90–101.

83. Drawz SM, Bonomo RA. Three Decades of β-Lactamase Inhibitors. Clin Microbiol Rev. 2010;23(1):160–201.

84. Cantón R. Antibiotic resistance genes from the environment: a perspective through newly identified antibiotic resistance mechanisms in the clinical setting. Clin Microbiol Infect. 2009;15:20–5.

85. Zong Z, Partridge SR, Thomas L, Iredell JR. Dominance of *bla*$_{CTX-M}$ within an Australian Extended-Spectrum -Lactamase Gene Pool. Antimicrob Agents Chemother. 2008;52(11):4198–202.

86. Cornaglia G, Giamarellou H, Rossolini GM. Metallo-β-lactamases: a last frontier for β-lactams? Lancet Infect Dis. Elsevier Ltd; 2011;11(5):381–93.

87. Walsh TR. Emerging carbapenemases: a global perspective. Int J Antimicrob Agents. 2010;36(S3):S8–14.

88. Docquier J-D, Lamotte-Brasseur J, Galleni M, Amicosante G, Frère J-M, Rossolini GM. On functional and structural heterogeneity of VIM-type metallo-β-lactamases. J Antimicrob Chemother. 2003;51(2):257–66.

89. Lauretti L, Riccio ML, Mazzariol A, Cornaglia G, Amicosante G, Fontana R, et al. Cloning and characterization of *bla*$_{VIM}$, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob Agents Chemother. 1999;43(7):1584–90.

90. Bennett PM. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. Br J Pharmacol. 2008;153:S347–57.

91. Fluit a C, Schmitz F-J. Resistance integrons and super-integrons. Clin Microbiol Infect. 2004;10(4):272–88.

92. Harbottle H, Thakur S, Zhao S, White DG. Genetics of antimicrobial resistance. Anim Biotechnol. 2006;17(2):111–24.

93. Carattoli A. Importance of integrons in the diffusion of resistance. 2001;32:243–59.

94. Barlow RS, Pemberton JM, Desmarchelier PM, Gobius KS. Isolation and characterization of integron-containing bacteria without antibiotic selection. Antimicrob Agents Chemother. 2004;48(3):838–42.

95. Rowe-Magnus D a., Mazel D. Integrons: Natural tools for bacterial genome evolution. Curr Opin Microbiol. 2001;4(5):565–9.

96. Bouvier M, Ducos-Galand M, Loot C, Bikard D, Mazel D. Structural features of single-stranded integron cassette attC sites and their role in strand selection. PLoS Genet. 2009;5(9):1–14.

97. Cambray, G, Guerout, A-M, Mazel D. Integrons. Annu Rev Genet. 2010;44(1):141–66.

98. Barraud O, Baclet MC, Denis F, Ploy MC. Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. J Antimicrob Chemother. 2010;65(8):1642–5.

99. Stalder T, Barraud O, Jové T, Casellas M, Gaschet M, Dagot C, et al. Quantitative and qualitative impact of hospital effluent on dissemination of the integron pool. ISME J. 2014;8(4):768–77.

100. Chen T, Feng Y, Yuan JL, Qi Y, Cao YX, Wu Y. Class 1 integrons contributes to antibiotic resistance among clinical isolates of *Escherichia coli* producing extended-spectrum beta-lactamases. Indian J Med Microbiol. 2013;31(4):385–9.

101. Rodrigues A. Mensagem do Presidente [Internet]. Centro Hospitalar Baixo Vouga. 2013 [cited 2005 May 20]. Available from: http://www.hip.min-saude.pt/Paginas/MensagemdoPresidente.aspx

102. Ribeiro JVB. Capacidade de Nitrificação e Desnitrificação da ETAR Norte-SIMRIA. Universidade de Aveiro. 2013.

103. Weisburg WG, Barns SM, Pelletier D a., Lane DJ. 16S Ribosomal DNA Amplification for Fhylogenetic Study. J Bacteriol. 1991;173(2):697–703.

104. Muyzer G, De Waal EC, Uitterlinden a. G. Profiling of Complex microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Ceaction-amplified Genes Coding for 16S rRNA. Appl Environ Microbiol. 1993;59(3):695–700.

105. Fromin N, Hamelin J, Tarnawski S, Roesti D, Jourdain-Miserez K, Forestier N, et al. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environ Microbiol. 2002;4(11):634–43.

106. Colomer-Lluch M, Jofre J, Muniesa M. Antibiotic Resistance Genes in the Bacteriophage DNA Fraction of Environmental Samples. PLoS One. 2011;6(3).

107. Yang Y, Zhang T, Zhang XX, Liang DW, Zhang M, Gao DW, et al. Quantification and characterization of β-lactam resistance genes in 15 sewage treatment plants from East Asia and North America. Appl Microbiol Biotechnol. 2012;95:1351–8.

108. Jiang L, Hu X, Xu T, Zhang H, Sheng D, Yin D. Prevalence of antibiotic resistance genes and their relationship with antibiotics in the Huangpu River and the drinking water sources, Shanghai, China. Sci Total Environ. 2013;458-460:267–72.

109. Falcone M, Mezzatesta ML, Perilli M, Forcella C, Giordano A, Cafiso V, et al. Infections with VIM-1 Metallo-β-lactamase-producing *Enterobacter cloacae* and their correlation with clinical outcome. J Clin Microbiol. 2009;47(11):3514–9.

110. Alves MS, Pereira A, Araújo SM, Castro BB, Correia ACM, Henriques I. Seawater is a

reservoir of multi-resistant *Escherichia coli*, including strains hosting plasmid-mediated quinolones resistance and extended-spectrum beta-lactamases genes. Front Microbiol. 2014;5(426):1–10.

111. Santos C, Caetano T, Ferreira S, Ramalheira E, Mendo S. A novel complex class 1 integron found in a Klebsiella pneumoniae isolate from Portugal. Clin Microbiol Infect. 2011;17(7):1036–9.

112. Borjesson S, Dienues O, Jarnheimer P-A, Olsen B, Matussek A, Lindgren P-E. Quantification of genes encoding resistance to aminoglycosides, beta-lactams and tetracyclines in wastewater environments by real-time PCR. Int J Environ Health Res. 2009;19(3):219–30.

113. Postollec F, Falentin H, Pavan S, Combrisson J, Sohier D. Recent advances in quantitative PCR (qPCR) applications in food microbiology. Food Microbiol. Elsevier Ltd; 2011;28(5):848–61.

114. Zhang T, Zhang M, Zhang XX, Fang HH. Tetracycline Resistance Genes and Tetracycline Resistant Lactose-Fermenting *Enterobacteriaceae* in Activated Sludge of Sewage Treatment Plants. Environ Sci Technol. 2009;43(10):3455–60.

115. Czekalski N, Gascón Díez E, Bürgmann H. Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. ISME J. 2014;8:1381–90.

116. Pei R, Kim S-C, Carlson KH, Pruden A. Effect of River Landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). Water Res. 2006;40(12):2427–35.

117. Liu XC, Zhang Y, Yang M, Wang ZY, Lv WZ. Analysis of bacterial community structures in two sewage treatment plants with different sludge properties and treatment performance by nested PCR-DGGE method. J Environ Sci. 2007;19(1):60–6.

118. Rozen Y, Belkin S. Survival of enteric bacteria in seawater. FEMS Microbiol Rev. 2001;25(5):513–29.

119. Zaitlin B, Watson SB. Actinomycetes in relation to taste and odour in drinking water: Myths, tenets and truths. Water Res. 2006;40(9):1741–53.

120. Heuer H, Krsek M, Baker P, Smalla K, Wellington EMH. Analysis of Actinomycete Communities by Specific Amplification of Genes Encoding 16S rRNA and Gel-Electrophoretic Separation in Denaturing Gradients. Appl Environ Microbiol. 1997;63(8):3233–41.

121. Ahmed SS, Alp E, Ulu-Kilic A, Doganay M. Establishing molecular microbiology facilities in developing countries. J Infect Public Health. 2015;8(6):513–25.

122. Miller JM, Astles R, Baszler T, Chapin K, Carey R, Garcia L, et al. Guidelines for safe work practices in human and animal medical diagnostic laboratories. Recommendations of a CDC-convened, Biosafety Blue Ribbon Panel. Morb Mortal Wkly Rep. 2012;61:1–102.

123. Verlicchi P, Al Aukidy M, Galletti A, Petrovic M, Barceló D. Hospital effluent: Investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. Sci Total Environ. Elsevier B.V.; 2012;430:109–18.

124. Boillot C, Bazin C, Tissot-Guerraz F, Droguet J, Perraud M, Cetre JC, et al. Daily physicochemical, microbiological and ecotoxicological fluctuations of a hospital effluent according to technical and care activities. Sci Total Environ. 2008;403(1-3):113–29.

125. Varela AR, André S, Nunes OC, Manaia CM. Insights into the relationship between antimicrobial residues and bacterial populations in a hospital-urban wastewater treatment plant system. Water Res. 2014;54:327–36.

126. Shannon CE, Weaver W. The mathematical theory of communication. Urbana: University of Illinois Press; 1963.

127. Pielou EC. The measurement of diversity in different types of biological collections. J Theor Biol. 1967;15(1):177.

128. Moura A, Tacão M, Henriques I, Dias J, Ferreira P, Correia A. Characterization of bacterial diversity in two aerated lagoons of a wastewater treatment plant using PCR – DGGE analysis. Microbiol Res. 2009;164:560–9.

129. Ramette A. Multivariate analyses in microbial ecology. FEMS Microbiol Ecol. 2007;62(2):142–60.

130. ter Braak CJF, Verdonschot PFM. Canonical correspondence analysis and related multivariate methods in aquatic ecology. Aquat Sci Across Boundaries. 1995;57(3):255–89.

131. Stokes HW, Gillings MR. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. FEMS Microbiol Rev. 2011;35(5):790–819.

132. El Salabi A, Walsh TR, Chouchani C. Extended spectrum β-lactamases, carbapenemases and mobile genetic elements responsible for antibiotics resistance in Gram-negative bacteria. Crit Rev Microbiol. 2012;39(2):113–22.

133. Hawkey PM, Jones AM. The changing epidemiology of resistance. J Antimicrob Chemother. 2009;64(Suppl 1):i3–10.

134. Lupo A, Coyne S, Berendonk TU. Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. Front Microbiol. 2012;3(18):1–13.

135. Diwan V, Chandran SP, Tamhankar AJ, Stalsby Lundborg C, Macaden R. Identification of extended-spectrum β-lactamase and quinolone resistance genes in *Escherichia coli* isolated from hospital wastewater from central India. J Antimicrob Chemother. 2012;67(4):857–9.

136. Chagas TPG, Seki LM, Cury JC, Oliveira J a L, Dávila a. MR, Silva DM, et al. Multiresistance, beta-lactamase-encoding genes and bacterial diversity in hospital

wastewater in Rio de Janeiro, Brazil. J Appl Microbiol. 2011;111(3):572–81.

137. Korzeniewska E, Korzeniewska A, Harnisz M. Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. Ecotoxicol Environ Saf. Elsevier; 2013;91:96–102.

138. Gündoğdu A, Jennison AV, Smith HV, Stratton H, Katouli M. Extended-spectrum β-lactamase producing *Escherichia coli* in hospital wastewaters and sewage treatment plants in Queensland, Australia. Can J Microbiol. 2013;59(11):737–45.

139. Yong D, Choi YS, Roh KH, Kim K, Park YH, Yum JH, et al. Increasing Prevalence and Diversity of metallo- β-lactamases in *Pseudomonas* spp ., *Acinetobacter* spp ., and *Enterobacteriaceae* from Korea. Antimicrob Agents Chemother. 2006;50(5):1884–6.

140. Lee K, Lee WG, Uh Y, Ha GY, Cho J, Chong Y, et al. VIM- and IMP-type metallo-β-lactamase-producing *Pseudomonas* spp. and *Acinetobacter* spp. in Korean hospitals. Emerg Infect Dis. 2003;9(7):868–71.

141. Libisch B, Watine J, Balogh B, Gacs M, Muzslay M, Szabó G, et al. Molecular typing indicates an important role for two international clonal complexes in dissemination of VIM-producing *Pseudomonas aeruginosa* clinical isolates in Hungary. Res Microbiol. 2008;159(3):162–8.

142. Marinescu F, Marutescu L, Savin I, Lazar V. Antibiotic resistance markers among Gram-negative isolates from wastewater and receiving rivers in South Romania. Rom Biotechnol Lett. 2015;20(1):10055–69.

143. Scotta C, Juan C, Cabot G, Oliver A, Lalucat J, Bennasar A, et al. Environmental microbiota represents a natural reservoir for dissemination of clinically relevant metallo-β-lactamases. Antimicrob Agents Chemother. 2011;55(11):5376–9.

144. Deng Y, Bao X, Ji L, Chen L, Liu J, Miao J, et al. Resistance integrons: class 1, 2 and 3 integrons. Ann Clin Microbiol Antimicrob. BioMed Central; 2015;14(45):1–11.

145. Gillings MR, Gaze WH, Pruden A, Smalla K, Tiedje JM, Zhu Y-G. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 2015;9(6):1269–79.

146. Hardwick S a., Stokes HW, Findlay S, Taylor M, Gillings MR. Quantification of class 1 integron abundance in natural environments using real-time quantitative PCR. FEMS Microbiol Lett. 2008;278(2):207–12.

147. Gaze WH, Abdouslam N, Hawkey PM, Wellington EMH. Incidence of class 1 integrons in a quaternary ammonium compound-polluted environment. Antimicrob Agents Chemother. 2005;49(5):1802–7.

148. Goldstein C, Lee MD, Sanchez S, Phillips B, Register B, Grady M, et al. Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock , companion animals, and exotics. Antimicrob Agents Chemother. 2001;45(3):723–6.

149. Rosser SJ, Young HK. Identification and characterization of class 1 integrons in

bacteria from an aquatic environment. The Journal of Antimicrobial Chemotherapy. 1999. p. 11–8.

150. Valasek MA, Repa JJ. The power of real-time PCR. Adv Physiol Educ. 2005;29:151–9.

151. Miranda CC, de Filippis I, Pinto LH, Coelho-Souza T, Bianco K, Cacci LC, et al. Genotypic characteristics of multidrug-resistant Pseudomonas aeruginosa from hospital wastewater treatment plant in Rio de Janeiro, Brazil. J Appl Microbiol. 2015;118(6):1276–86.

152. Henriques IS, Fonseca F, Alves A, Saavedra MJ, Correia A. Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from estuarine waters. Res Microbiol. 2006;157:938–47.

153. Invitrogen. Real-time PCR: from theory to practice. Carlsbad, California, Invitrogen Corporation; 2008.

154. Reinthaler F., Posch J, Feierl G, Wüst G, Haas D, Ruckenbauer G, et al. Antibiotic resistance of *E. coli* in sewage and sludge. Water Res. 2003;37(8):1685–90.

155. Baquero F, Martínez J-L, Cantón R. Antibiotics and antibiotic resistance in water environments. Curr Opin Biotechnol. 2008 Jun;19(3):260–5.

156. Joss A, Keller E, Alder AC, Gobel A, McArdell CS, Ternes T, et al. Removal of pharmaceuticals and fragrances in biological wastewater treatment. Water Res. 2005;39(14):3139–52.

157. Verlicchi P, Al Aukidy M, Zambello E. What have we learned from worldwide experiences on the management and treatment of hospital effluent? — An overview and a discussion on perspectives. Sci Total Environ. 2015;514:467–91.

158. Rodriguez-Mozaz S, Chamorro S, Marti E, Huerta B, Gros M, Sànchez-Melsió A, et al. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. Elsevier Ltd; 2015;69:234–42.

159. Li D, Yang M, Hu J, Zhang J, Liu R, Gu X, et al. Antibiotic-resistance profile in environmental bacteria isolated from penicillin production wastewater treatment plant and the receiving river. Environ Microbiol. 2009;11(6):1506–17.

160. Graham DW, Olivares-Rieumont S, Knapp CW, Lima L, Werner D, Bowen E. Antibiotic resistance gene abundances associated with waste discharges to the Almendares River near Havana, Cuba. Environ Sci Technol. 2011;45(2):418–24.

161. Laht M, Karkman A, Voolaid V, Ritz C, Tenson T, Virta M, et al. Abundances of tetracycline, sulphonamide and Beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load. PLoS One. 2014;9(8):1–8.

162. Xi C, Zhang Y, Marrs CF, Ye W, Simon C, Foxman B, et al. Prevalence of antibiotic resistance in drinking water treatment and distribution systems. Appl Environ

Microbiol. 2009;75(17):5714–8.

163. Walsh TR, Toleman MA, Poirel L, Nordmann P. Metallo-β-lactamases: the quiet before the Storm? Clin Microbiol Infect. 2005;18(2):306–25.

164. Van Der Bij a. K, Van Mansfeld R, Peirano G, Goessens WHF, Severin J a., Pitout JDD, et al. First outbreak of VIM-2 metallo-β-lactamase-producing *Pseudomonas aeruginosa* in the Netherlands: Microbiology, epidemiology and clinical outcomes. Int J Antimicrob Agents. 2011;37(6):513–8.

165. Giakkoupi P, Xanthaki A, Kanelopoulou M, Vlahaki A, Kontou S, Papafraggas E, et al. VIM-1 metallo-β-lactamase-Producing *Klebsiella pneumoniae* strains in Greek Hospitals. J Clin Microbiol. 2003;41(3):3893–6.

166. Tsakris A, Ikonomidis A, Pournaras S, Tzouvelekis LS, Sofianou D, Legakis NJ, et al. VIM-1 metallo-β-lactamase in *Acinetobacter baumannii*. Emerg Infect Dis. 2006;12(6):981–3.

167. Weile J, Rahmig H, Gfroer S, Schroeppel K, Knabbe C, Susa M. First detection of a VIM-1 metallo-β-lactamase in a carbapenem-resistant *Citrobacter freundii* clinical isolate in an acute hospital in Germany. Scand J Infect Dis. 2007;39(3):264–6.

168. Quinteira S, Peixe L. Multiniche screening reveals the clinically relevant metallo-β-lactamase VIM-2 in *Pseudomonas aeruginosa* far from the hospital setting: an ongoing dispersion process? Appl Environ Microbiol. 2006;72(5):3743–5.

169. Verlicchi P, Al Aukidy M, Zambello E. Occurrence of pharmaceutical compounds in urban wastewater: Removal, mass load and environmental risk after a secondary treatment—A review. Sci Total Environ. 2012;429:123–55.

170. Ebner P. Class 1 integrons in various *Salmonella enterica* serovars isolated from animals and identification of genomic island SGI1 in *Salmonella enterica* var. Meleagridis. J Antimicrob Chemother. 2004;53(6):1004–9.

171. van Essen-Zandbergen A, Smith H, Veldman K, Mevius D. Occurrence and characteristics of class 1, 2 and 3 integrons in *Escherichia coli*, *Salmonella* and *Campylobacter* spp. in the Netherlands. J Antimicrob Chemother. 2007;59:746–50.

172. Ma L, Zhang X-X, Zhao F, Wu B, Cheng S, Yang L. Sewage treatment plant serves as a hot-spot reservoir of integrons and gene cassettes. J Environ Biol. 2013;34(April):307–14.

173. Du J, Ren H, Geng J, Zhang Y, Xu K, Ding L. Occurrence and abundance of tetracycline, sulfonamide resistance genes, and class 1 integron in five wastewater treatment plants. Environ Sci Pollut Res. 2014;21:7276–84.

174. Batt AL, Bruce IB, Aga DS. Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. Environ Pollut. 2006;142(2):295–302.

175. Brown KD, Kulis J, Thomson B, Chapman TH, Mawhinney DB. Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and

the Rio Grande in New Mexico. Sci Total Environ. 2006;366:772–83.

176. Chen H, Zhang M. Occurrence and removal of antibiotic resistance genes in municipal wastewater and rural domestic sewage treatment systems in eastern China. Environ Int. Elsevier Ltd; 2013;55:9–14.

177. Pruden A, Pei R, Storteboom H, Carlson KH. Antibiotic resistance genes as emerging contaminants: studies in Northern Colorado. Environ Sci Technol. 2006;40(23):7445–50.

178. Zhang T, Fang HHP. Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. Appl Microbiol Biotechnol. 2006;70:281–9.

Appendix 1 – DNA Extraction

DNA extraction was performed with the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania), according with the manufacturer's instructions.

Thus, 400 µL of the Lysis Solution were added to the mixture, followed by incubation at 65 ºC for 5 min. At this time, 600 µL of chloroform were added and by inversion the solution gently emulsified. The samples were centrifuged for 10 min at 13000rpm and the upper aqueous phase was transferred to a clean and sterile microtube. 1 volume of isopropanol was added and mixed gently by inversion, followed by an incubation of 15 min ate 4 ºC. The mixture was centrifuged at 13000 rpm for 10 min and the supernatant completely removed. The pellet constituting the genomic DNA was gently dissolved in 100 µL of the provided NaCl solution (1 M), until the pellet was completely dissolved. 2,5 volumes of cold ethanol were added and the genomic DNA was allowed to precipitate 10 min at -20 ºC. After centrifugation for 5 min at 13000 rpm, the ethanol was removed and the pellet washed with 250 µL of 70 % ethanol. This solution was removed by centrifugation 5 min at 13000 rpm and the pellet was allowed to dry completely at 50 ºC for 2 min. Finally, the extracted DNA was dissolved in 100 µL of TE stored at -20 ºC until further analysis.

Appendix 2 –Agarose Gels handling

The analysis of DNA and PCR products were generally performed on agarose gel electrophoresis. The samples were mixed with 6X loading buffer in a proportion of 1:6 (v/v) and loaded in a 1 % agarose gel. The gel was prepared with 1X of TAE buffer (Bio-Rad) and EtBr (AppliChem) to a final concentration of 0.5 µg/mL added before pouring the melted agarose in the running tray. In all gels a DNA marker was included, either 0.5 µg of the DNA Ladder Mix (Fermentas). Electrophoresis was generally performed at 120 V for the desired time (normally 30 min) or at 100 V during 50 min and the DNA was analysed under UV light and the image acquired in the ATTO image acquisition system.

Solutions:

Loading buffer 6X: 2.5 mg/mL of bromophenol blue, 2.5 mg/mL of xylene cyanol FF and 30 % (v/v) glycerol; stored at 4 ºC.

Appendix 3 - Purification and concentration of PCR products

Purifications of PCR products were performed using the NZYGelpure (NZYtech), according to the manufacturer's instructions.

The volume of the reaction mixture was transferred to a 1.5 mL microcentrifuge tube and five volumes of Binding Buffer were added and mixed well. This mixture was applied to an NZYTech spin column, incubated at room temperature for 2 min and centrifuged for 1 min at top speed. The flow-through was discarded and 600 µL of Wash Buffer were added to the spin column. After 2 min of room temperature incubation, the column was centrifuge for 1 min and the flow-through was discarded. An additional 1 min centrifugation was performed to remove residual ethanol. The NZYTech spin column was then placed into a clean 1.5 mL microcentrifuge tube and 30 to 50 µL of sterile distilled water were added to the center of the column. The DNA-containing column was incubated at room temperature for 2 min and then centrifuged for 1 min to elute the DNA. The sample was stored at -20 °C until further use.

Appendix 4 – Preparation and transformation of chemically competent *E. coli* cells

i. Preparation of competent cells by calcium-chloride method

Chemically competent cells were prepared using and adaptation of the procedure described by Sambrook and Russell (2001). The desired strain was inoculated in 10 mL of LB medium supplemented with the appropriated selective marker, overnight at 37 °C, 180 rpm. The pre-culture (500 µL) was used to inoculate 50 mL of fresh LB medium supplemented with the appropriate antibiotic. The bacterial culture was grown at 37 °C, 180 rpm, to an OD600nm of approximately 0.3 and centrifuged at 4 °C for 2 min at 6300 x g. The resulting pellet was washed with 13 mL of ice-cold 0.1 M MgCl2 and centrifuged as mentioned before. The cells were washed again with 25 mL of 0.1 M CaCl2 solution, incubated on ice for 20 min and centrifuged as above. Finally the cells were resuspended in 1 mL of cryopreservation buffer (CaC_{l2} 0.1 M, 15% (v/v) glycerol), 50 µL were distributed in 1.5 mL microcentrifuge tubes and stored at -80 °C until use.

ii. Transformations

An aliquot of 50 µL of competent *E. coli* DH5α cells stored at -80 °C were thawed on ice and the DNA was added (~5-100 ng of plasmid DNA or 5 µL of ligation reaction). The mixture was incubated in the 1.5 mL microcentrifuge tube on ice for 30 min and transferred to 42 °C for 45 sec. The microcentrifuge tube was directly placed on ice and 250 µL of LB medium was added. The cells were grown for 1 hour at 37 °C, 180 rpm. Afterwards, approximately 100 µL of the transformation were finally spread on LB agar containing 100 µg/mL of ampicillin, 100 µg/mL of X-Gal and 0.5 mM of IPTG. The plates were incubated at 37 °C overnight.

Appendix 5 – Extraction of plasmid DNA

The extraction of plasmid DNA from *E. coli* was performed with the Miniprep NZYtech Kit, according with manufacturer's instructions.

Briefly, a bacterial culture was grown in LB medium with the appropriate selection agent, overnight at 37 ºC with aeration (180 rpm). 5 mL of this culture was centrifuged at 11000 x g for 2min, the supernatant was discharged and the cells were completely resuspended in 250 µL of Resuspension Solution (A1). 250 µL Lysis Buffer (A2) were added and the tube was gently inverted until the solution became clear and viscous, followed for an incubation at room temperature for a maximum of 4 min. Neutralization was performed by the addition of 300 µL of the Neutralization Buffer (A3) and by the solution was mixed by inverting softly the tube and centrifuged for 10 min at room temperature. An NZYTech spin column was placed in a 2 mL collecting tube. The supernatant was transferred onto the column, avoiding the transfer of the white precipitate and centrifuged for 1 min at 11000 x g. The flow-through was discarded and the column was placed back into the same collection tube. The DNA attached to the column was washed by the addition of 500 µL of Wash Buffer (AY) followed by centrifugation for 1 min at 11000 x g and the flow-through was discarded. Another wash was performed by adding 600 µL of another Wash Buffer (A4, with ethanol previously added) followed by centrifugation for 1 min at 11000 x g and the flow-through was once more discarded. Finally the column was transferred into a sterile 1.5 mL microcentrifuge tube and centrifuged for 2 min at the same speed to remove residual ethanol.

The plasmid DNA was eluted by the addition of 50 µL of Elution Buffer (AE) to the center of the column, incubation at room temperature for 1 min at room temperature and centrifugation at 11000 x g for another 1 min.

Appendix 6 - DNA concentration – Qubit® (Invitrogen)

The Quant-iT™ working solution was made by diluting the Quant-iT™ reagent 1:200 in Quant-iT™ buffer (DNA or RNA reagent and buffer according to the sample to be measured). 199 µL of the working solution were loaded into the assay tubes and 1 µL of sample was added (the final volume must be 200 µL). The mixture was mixed by vortexing 2-3 s and incubated for 2 min at room temperature. The tube was then inserted into the Qubit™ fluorometer and the concentration was calculated following the instructions on the screen.