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Occurrence of antimicrobial pharmaceuticals and characterization of β-lactamases in Gram-negative pathogens from wastewater

PhD thesis

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Abbreviations

AME	Aminoglycoside modifying enzymes
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
AR	Antibiotic resistance
ARB	Antibiotic resistant bacteria
ARGs	Antibiotic resistance genes
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BLI	Beta-lactamase inhibitor
Bp	Base pair
CF	Cystic fibrosis
CDS	Complete coding sequence
CFU	Colony forming unit
CAN	Cetrimid nalidixic agar
DDD	Defined daily dose
DNA	Deoxyribonucleic acid
EARS-Net	European Antimicrobial Resistance Surveillance Network
EDTA	Ethylene diamine tetraacetic acid
EEA	European Economic Area
EEC	European Economic Community
EMA	European Medicines Agency
EMB	Eosin methylene blue
ESAC-Net	European Surveillance of Antimicrobial Consumption Network
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GO	Gene ontology
H1-H4	Hospital effluent
HCCA	Alpha-cyano-4-hydroxycinnamic acid
HGT	Horizontal gene transfer
LC-MS	Liquid chromatography mass spectrometry
LTCFs	Long term care facilities
MALDI-TOF/MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
MAR	Multiple antimicrobial resistance
MDR	Multiple drug resistance
MRR	Multi-resistance region
MS	Mass spectrometry
MSP	Main spectra
MUM	Maximal Unique Matches

NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
NH	Nursing home
OMPs	Outer membrane proteins
PBPs	Penicillin-binding proteins
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Potential Hydrogen
PMQR	Plasmid-mediated quinolone resistance
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SPE	Solid phase extraction
TAE	Tris acetic acid disodium EDTA buffer
UHPLC-MS	Ultra-high performance liquid chromatography mass spectrometry
UNSDG	United Nations Sustainable Development Goals
UV	Ultra violet radiation
UWW	Urban wastewater
WTP	Water treatment plant
WWTP	Wastewater treatment plant
WHO	World Health Organization
XDR	Extra drug resistant

Antibiotics and antibiotic resistant genes

CAZ	Ceftazidime
CIP	Ciprofloxacin
CPD	Cefpodoxime
CRO	Ceftriaxone
CTX	Cefotaxime
FOX	Cefoxitin
GN	Gentamicin
IMP	Imipenem
MEM	Meropenem
SXT	Sulfamethoxazole/trimethoprim
bla	Beta-lactamase
CTX-M	Cefotaxime-Munich
ESBL	Extended spectrum β-lactamase
GIM	Germany imipenemases
IMP	Imipenem-resistant pseudomonas
KPC	*Klebsiella pneumoniae* carbapenemase

MBL	Metallo-β-lactamase
NDM	New Delhi metallo-β-lactamase
OXA	Oxacillinases
SHV	Sulfhydryl variable
SPM	São Paulo metallo-β-lactamase
TEM	Temoneira
VIM	Verona integron encoded metallo-β-lactamase

SUMMARY

The antibiotics frequently administered to animals and humans persist in the environment through accumulation, transformation, and deposition. They are regarded as emergent micropollutants of concern whose long-term exposure can produce significant eco-toxicological effects even at minute concentrations. The occurrence of these antimicrobial pharmaceuticals, their metabolites and diverse bacteria in aquatic media has the potential to select for resistant bacteria. According to Directive 91/271/EEC, no specific restrictions are imposed for hospital sewage effluents that can be discharged into urban wastewater sewerage networks without previous treatment. This implies that many countries across Europe release untreated hospital effluents carrying antimicrobials into the urban wastewater stream for co-treatment with the municipal wastewater at the urban wastewater treatment plant prior to discharge into the environment. The antimicrobials in the wastewater systems may exert a continuous selective pressure leading to the evolution of antibiotic resistant bacteria through adaptation, vertical and horizontal transfer of resistance determinants. Some of these bacteria such as, *Escherichia coli*, *Klebsiella* species and *Enterobacter* form part of bowel microbiota and are human opportunistic pathogens, while others such as *Pseudomonas aeruginosa* is an environmental organism that is associated with hospital-acquired infections. These bacteria have developed resistance to multiple antimicrobials, including enzymatic degradation of the broad-spectrum β-lactams that are commonly used in clinical practice. Among the most common degrading enzymes are acquired extended-spectrum serine β-lactamases (ESBLs), which inactivate the oxyimino cephalosporins (ceftriaxone, ceftazidime, cefepime) and aminopenicillins (amoxicillin and ampicillin), as well as metallo-β-lactamases (MBLs), which degrade most of the β-lactams, including the lifesaving carbapenems.

The main aim of this study was to determine the presence and concentration of selected antibiotic compounds in municipal wastewater mainly from human sources and to isolate ESBL and carbapenemase-producing Gram-negative opportunistic pathogens, particularly some members of the order Enterobacterales (*Klebsiella* species, *E. coli*, and *Enterobacter* species), and *P. aeruginosa* from hospital effluents, urban wastewater and wastewater treatment plant (influent, activated sludge reactor and digested sludge), and to characterize other plasmid-mediated mechanisms conferring resistance to multiple antibiotics among the isolates. Eight antibiotics

belonging to four antimicrobial pharmaceuticals namely macrolides (azithromycin, clarithromycin), fluoroquinolones (ciprofloxacin, norfloxacin, and ofloxacin), sulfonamides (sulfadiazine, sulfamethoxazole), and trimethoprim were recovered at concentrations that varied significantly ($P < 0.05$) among the wastewater samples. The highest concentration detected was that of trimethoprim (24907 ngL^{-1}), ofloxacin (21916 ngL^{-1}), and ciprofloxacin (11710 ngL^{-1}). Sulfadiazine was the lowest detectable antibiotic, whose concentration ranged between 5.5 ngL^{-1} and 38.5 ngL^{-1}. Whereas the concentration of trimethoprim and ciprofloxacin was significantly higher in hospital samples ($P = 0.012$, and $P = 0.025$, respectively), that of sulfadiazine was significantly higher ($P = 0.008$) in the wastewater treatment plant and the urban wastewater samples compared to hospital effluent samples. The study established multiple antibiotic resistance (MAR) in both Enterobacterales (65.08%, n = 126) and *P. aeruginosa* (72.85%, n = 151). Multiple antibiotic resistance in the wastewater treatment plant was observed to increase from the activated sludge reactor (MAR index 0.508) to the digested sludge (MAR index 0.560) for Enterobacterales and from MAR index 0.84 to MAR index 0.88 in *P. aeruginosa*. Among the enteric bacteria, 86% (n = 50), of *E. coli* isolates, 77% (n = 33), of *Klebsiella* isolates and 25% (n = 4) of *Citrobacter* species isolates phenotypically expressed extended spectrum β-lactamase. The ESBL gene, $bla_{\text{CTX-M-27}}$ was found in *E. coli*, while *Klebsiella* species harbored $bla_{\text{CTX-M-15}}$, $bla_{CTX\text{-}M\text{-}30}$, or $bla_{\text{SHV-12}}$. Genes coding for aminoglycoside-modifying enzymes, adenylyltransferases (*aadA1*, *aadA5)*, phosphotransferases (*aph(6)-1d, aph(3")-Ib)*, acetyltransferases (*aac(3)-IIa)*, (*aac(6)-Ib*), sulfonamide/trimethoprim-resistant dihydropteroate synthase (*sul*), dihydrofolate reductase (*dfrA*), and plasmid-mediated quinolone resistance protein (*qnrB1*) were also identified among the Enterobacterales. 66% (n = 57) of carbapenem-resistant *P. aeruginosa* were metallo-β-lactamase producers. Plasmid DNA sequences confirmed $bla_{VIM\text{-}4}$ as the main variant circulating among the *P. aeruginosa* isolates from the wastewater environment. The findings of this study offer supporting guidance to assess the human health risks posed by environmental exposure to antimicrobial pharmaceuticals and antibiotic-resistant bacteria and contribute to important knowledge that is applicable in planning effective strategies to minimize the spread of multiresistance in the environment.

1.0 INTRODUCTION

One of the major milestones of the last century was the advent of antimicrobial pharmaceuticals, which are currently widely applied in human and veterinary medicine to prevent and manage infections, and in animal husbandry as growth promoters (Cycoń et al. 2019). Antibiotics are a class of active pharmaceutical compounds that are widely consumed around the world to inhibit bacterial proliferation through cell destruction or growth inhibition (Kümmerer 2009). Data from scientific literature, national and regional surveillance systems from numerous countries over time indicate a steadily increasing antibiotic use worldwide (30%), primarily due to rising demand in low and middle-income countries (Gelband et al. 2015). This unprecedented increase in antibiotic use continues to raise concern about their potentially harmful effects on the environment (Bengtsson et al. 2018). However, despite their potential environmental and health effects, the use of these agents has revolutionized health care by improving hygiene and considerably changing the outcome of bacterial infections, which has in turn, significantly increased the average expected lifespan (Carvalho and Santos 2016a, Chowdhury et al. 2017). Their consumption varies from region to region and from country to country (Göbel et al. 2005). Studies have shown that many of these antimicrobials are not completely metabolized during therapeutic use and an estimated 30%–90% end up being excreted as active substances into sewage water, resulting in the presence of multiple classes of antibiotics being widely detected in various urban wastewater treatment plants and the receiving environment around the world (Chen et al. 2006, Li 2014). Their consumption patterns influence the extent of their environmental contamination where an increase in consumption, especially during the cold season when the frequency of infections is higher, elevates their occurrence in environmental systems (Wang et al. 2020), which correlates with the emergence of multiresistant bacteria and their rapid expansion (Levy 2002). Due to their widespread application, antimicrobials have been and continue to be discharged into the environment via wastewater of human origin from different sources, including households (domestic), hospitals (clinical), veterinary and animal husbandry, and pharmaceutical factories (industrial) (Kemper 2008). Following their discharge into water systems, several antimicrobials and their by-products are detected in the environment at concentrations that range from ng L^{-1} to μg L^{-1} (Seifrtova et al. 2009). They reach the aquatic environment mainly through the flow of wastewater treatment plant effluent into surface water or into groundwater (Carvalho and Santos

2016b). They are considered emergent micropollutants with the potential to create selective pressure for the development of microbial resistance in the environment (Kümmerer 2009b, Kumar et al. 2019). The permissible limits of the widely used substances of priority concern which may pose potential risks in aqueous media, excluding antibiotics, were set out by the EU Directive 2013/39/EU within the European Union to maintain environmental quality standards and ecological integrity (Ricci et al. 2016). Upon examining various ecotoxicological reports, the multiple threats posed by antibiotics as environmental contaminants were, however, recognized and the EU, alongside other countries, introduced a regulatory framework to monitor emerging substances of concern in the aquatic environment (Wang et al. 2020). For instance, in its decision of 2015 (EU Decision, 2015/495 of March 20, 2015), the EU Commission established a watch list of three antibiotics belonging to the macrolide class, namely clarithromycin, azithromycin, and erythromycin as contaminants of priority concern due to their potential risk to the aquatic environment, and thereafter added amoxicillin and ciprofloxacin to the watch list in 2018 (EU Decision, 2018/840 of June 5, 2018) (Felis et al. 2020). The occurrence of antimicrobial compounds in the environment varies among the different antimicrobial classes depending on their frequency of usage, and a major concern about their presence in the environment relates to the emergence of antibiotic resistance genes (ARGs) and the evolution of antibiotic-resistant bacteria (ARB), which endanger pharmaceuticals' ability to control microbial pathogens (Kumar et al. 2019).

Antimicrobial resistance (AMR) presents a global challenge to the fight against infections in modern time (Commission 2017). Each year more than 670,000 infections are due to antibiotic resistant bacteria in the European Union/European Economic Area (EU/EEA) according to data from the European Antimicrobial Resistance Surveillance Network (EARS-Net), and approximately 33,000 people succumb to these infections (Cassini et al. 2019). It is projected that, close to 2.4 million people are likely to die globally in high-income countries by the year 2050 due to diseases caused by antibiotic resistant microorganisms (UN 2019). The widespread use of antimicrobials in clinical practice to control infectious diseases, their application in veterinary medicine coupled with the discharge of non-treated pharmaceutical effluent into the environment results in selective pressure which is associated with the emergence and subsequent evolution of bacteria resistant to antibiotics (Islam 2011). Bacteria have shown the ability to develop antimicrobial resistance in response to stressors in the environment in the form of bioactive

molecules, which include antibiotics, heavy metals, and disinfectants, among other biocides (Berendonk et al. 2015). For instance, hospital effluent carries high bacterial loads and might contain sub-lethal concentrations of antimicrobial agents and their metabolites that enter wastewater and can facilitate the emergence and spread of resistance genes among bacteria (Pärnänen et al. 2019). In addition, wastewater treatment plants (WWTPs) contribute to the progression and persistence of antimicrobial resistant bacteria in the environment worldwide (Galler et al. 2014). It has been demonstrated that wastewater treatment plants serve as sinks for high loads of antimicrobials, ARB, and their genetic resistance determinants (Rizzo et al. 2013b). They also provide optimal conditions, including pH and temperature, which facilitate lateral gene transfer, capable of transforming commensal bacteria into reservoirs of resistance genes (Kelly et al. 2009).

Members of the order Enterobacterales, which bear similar biochemical and genetic characteristics are ubiquitous and form a major part of gut microbiota (Partridge 2015). Some of them, such as *Klebsiella pneumoniae, E. coli, Proteus, Citrobacter,* and *Enterobacter* cause infections including in the urinary tract, bloodstream, and respiratory tract (hospital and health-care associated pneumonia), as well as intestinal and intra-abdominal infections (Pitout and Laupland 2008, Qin et al. 2008). Enterobacterales exhibit a wide range of resistance attributed to either mutations in chromosomal genes or mobile genes captured from different source species by various mobile genetic elements and transferred to plasmids, which can shuttle between cells and confer or enhance resistance to certain chemical classes of antimicrobials that are frequently used against multidrug-resistant microorganisms (Partridge 2015). Fluoroquinolones and β-lactam antibiotics, which include the sub-groups of penicillins, cephalosporins, and carbapenems are the most frequently prescribed antibiotics and preferred therapeutic choices against infections caused by members of Enterobacterales (Damoa-Siakwan 2005). Hospitals and other environments characterized by high amounts of antibiotics are associated with multidrug-resistant Gram-negative bacteria that have demonstrated an increasing resistance to those compounds (Hocquet et al. 2016). ARGs, such as genes coding for ESBL and carbapenemases harbored by Enterobacterales and other Gram-negative bacteria are clinically significant and have been reported from hospital effluents and WWTPs (Lamba et al. 2017, Haller et al. 2018). These genes are typically encoded on plasmids which harbor mobile genetic elements such as transposons or integrons and genes known to encode resistance to other antimicrobial agents (Szczepanowski et

al. 2009). ESBLs are β-lactamases that are capable of hydrolyzing broad-spectrum cephalosporins and aztreonam, whose activity is inhibited by clavulanic acid (Paterson and Bonomo 2005). More than 300 subtypes of ESBLs have been described and their evolution is believed to originate from common ancestral types: TEM-1, TEM-2, or SHV-1 (Bush and Jacoby 2010b). Mutations occurring in those genes resulted in new β-lactamases which can hydrolyze extended-spectrum cephalosporins and aztreonam (Slama 2008, Smet et al. 2008). Enterobacterales are also known to express ESBLs different from TEM, or SHV related types, such as CTX-M-type β-lactamases which are encoded by genes captured on transferable plasmids and are among the most widespread ESBLs in Europe (Coque et al. 2008b). Carbapenems (imipenem, doripenem, ertapenem, and meropenem) are the most potent antimicrobials used to manage life-threatening infections caused by multiresistant Gram-negative bacilli and their efficacy has been diminishing since carbapenem-resistant Gram-negative strains have emerged following their extensive use (Codjoe and Donkor 2018). Carbapenemase producers are resistant to almost all β-lactams and to other classes of antibiotics (Woodford et al. 2014). Their occurrence in the environmental matrices is increasing with hospital wastewater being reported as a key reservoir of carbapenemase-producing Enterobacterales (Zhang et al. 2012). On the other hand, *P. aeruginosa* which is not a commensal human bacterium is a widespread nosocomial pathogen commonly associated with life-threatening infections acquired in intensive care units including urinary tract, cystic fibrosis (CF), bone and joint infections, ventilator-associated pneumonia, burn and wound and soft tissue injuries, bacteremia and systemic infections (Bassetti et al. 2018, Burrows 2018, Parkins et al. 2018). This species is known for its metabolic versatility with the ability to utilize different organic compounds as carbon and energy sources as well as survival in the low nutrient environments, formation of biofilm in moist environments, production of a myriad of virulence factors, and intrinsic resistance to many antibiotics and antiseptics, ability to acquire further resistance mechanisms to multiple antimicrobial categories, all of which contribute towards its pathogenicity *(*Granato et al. 2018*).* The World Health Organization (WHO) has placed *P. aeruginosa* in the list of "critical" category of bacterial pathogens of priority concern which urgently require a treatment strategy, research, and development of new antibiotics (Tacconelli et al. 2018). This bacterium is widespread in wastewater, with higher concentrations in hospital effluents, and enters into other environments through discharges (Tuméo et al. 2008).

The development of antimicrobial resistance spurred by antibiotics and other stressors in the environment raises concern due to the likelihood of simultaneous transmission of virulence and resistance determinants to multiple antibiotic classes by mobile genetic elements in bacteria, which may directly or indirectly reach human and animal hosts. The progression of resistance in the environment presents a considerable challenge to the successful achievement of the One Health initiative envisaged by WHO as well as the full realization of the United Nations Sustainable Development Goals (UNSDG) related to water and sanitation. The anthropogenic activities that result in the discharge of antimicrobial pharmaceuticals into environmental matrices and the biological connectivity between human and environmental microbes whose evolution can be driven by those antimicrobials necessitate an approach that provides sufficient guidelines for mitigation measures to combat the spread of AMR in the environment and thus promote achievement of One Health initiative. Data on the prevalence of antimicrobial pharmaceuticals and antimicrobial resistance facilitated by β-lactamase producing multiresistant Gram-negative bacteria of clinical importance in wastewater from human sources in southwest Hungary is unavailable since most studies are centered on resistance in the clinical environment. This study was therefore aimed at bridging this knowledge gap by investigating the occurrence of antibiotics residues in wastewater from hospital's effluent and wastewater treatment plant as well as unearthing the prevalence of multiresistant Enterobacterales and *P. aeruginosa* in the same environment.

2.0 LITERATURE REVIEW

2.1 One Health Paradigm

The current momentum associated with the One Health concept during the past decade was driven initially by a series of strategic goals, known as the 'Manhattan Principles' which were derived during a 'One World, One Health' meeting of the Wildlife Conservation Society entitled Building Interdisciplinary Bridges to Health in a "Globalized World" held in September 2004 in New York. These goals, developed for combating threats to life on Earth, clearly recognized the link between human health, animal health and the environment, and the threats that diseases pose to economies.

The One Health concept is an integrative effort of multiple disciplines working locally, nationally, and globally to attain optimal health for people, animals and the environment (King et al. 2008). It is an approach that recognizes that the health of people is closely connected to the health of animals and our shared environment. One Health issues include emerging, re-emerging, and endemic zoonotic diseases, antimicrobial resistance, food safety and food security, environmental contamination, climate change and other health threats shared by people, animals, and the environment. For example antimicrobial-resistant microbes which can quickly spread through communities, the food supply, healthcare facilities, and the environment (soil, water), making it harder to treat certain infections in animals and people have been recognized as a threat to public health globally (WHO 2019). Many emergent pathogens are not only linked to increasing contact between humans and animals, both domestic and wild, but to climate, the environment especially, the need for clean drinking water, and to the expansion of international travel and trade. The role of the wildlife-livestock human-ecosystem interfaces has been fundamental to the development of the One Health paradigm over the past decade, a concept that recognizes that the health of humans, animals, and ecosystems are interconnected, and that to better understand and respond to zoonotic diseases requires coordinated, collaborative, multidisciplinary and cross-sectoral approaches (Mackenzie et al. 2014).

2.2 Occurrence of antibiotics in the environment

Antibiotics are massively used in human and veterinary medicine where they undergo structural change within the bodies of humans and animals before excretion resulting in metabolites. After excretion, both parent compounds and metabolites can undergo additional structural modifications by biotic and abiotic processes in technical facilities such as WWTPs and water treatment plants (WTPs), and the natural environment. Antibiotics and their by-products in the environment may persist through a cycle of partial transformation and bioaccumulation and gradual deposition in soil, surface water, and groundwater (Carvalho and Santos 2016a). Antibiotics and their by-products in aquatic environments can be absorbed by animals (food-producing animals and fishes) or reach the drinking water inducing possible long-term effects on humans as a continuous part of their diet through water or food at low concentrations (Manzetti and Ghisi 2014). They may reach the aquatic environment by the direct discharge of wastewater treatment plant effluent to surface water or to groundwater, landfill leachate, leaking sewers, and manure storage tanks or lagoons, runoff and leaching from farmland fertilized with manure, or through sewage disposal in agricultural areas and other discharges (Li 2014, Carvalho and Santos 2016a). **Figure 1** illustrates the pathways followed by antibiotics to circulate between their sources and the environment.

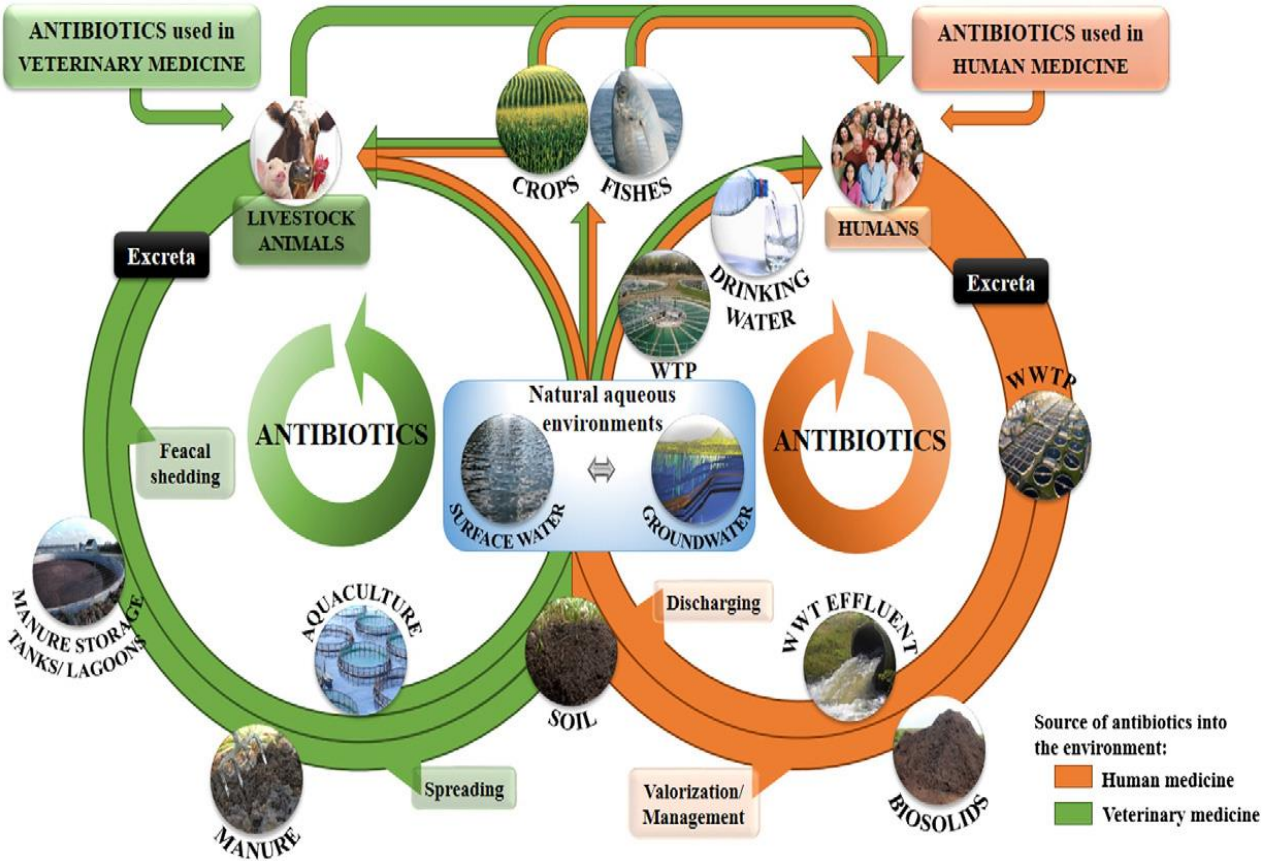


Figure 1. Pathways through which antibiotics consumed by humans and animals enter and circulate in the environment (Figure adapted from Carvalho and Santos 2016a).

Pharmaceuticals are widely used in livestock production and in agriculture, in addition to human use (Koch et al. 2021). Each year, approximately 24.6 million pounds of antibiotics are used in livestock farming (Van et al. 2020). This has become a global practice because low-dose antibiotics were found to boost animal and bird growth by adding them to animal feeds (Kumar et al. 2018). Their extensive use in animal production forms the main source of environmental antibiotics (Kinney and Heuvel 2020). Numerous studies have reported the presence of pharmaceutical compounds or their metabolites in the geosphere and biosphere (Bartrons and Penuelas 2017, Riaz et al. 2018), with pharmaceutical contaminants being reported in polar regions, the most pristine environment on earth (González-Alonso et al. 2017). Continuous antimicrobials exposure has seen more antibiotics become less effective due to the growing resistance observed among the primary and opportunistic pathogens, resulting in higher medical and economic costs and increased mortality (Zhen et al. 2019). It has been observed that the

concentrations of antibiotic classes vary based on the antimicrobial compound and environmental matrix and the load tends to decrease from wastewater generated by human activity to the surface and groundwater (Carvalho and Santos 2016a). The pattern and consumption rate, excretion, and the efficacy of elimination by wastewater treatment processes, together with weather conditions, especially rainwater, usually influence the concentrations of the antibiotics detected in WWTP influents and effluents (Osorio et al. 2012). Certain antimicrobial agents, especially macrolides, sulfonamides, quinolones, and trimethoprim, persist in the aqueous environment and are among the most frequently detected substances in the environmental matrices due to their stability and because they are frequently prescribed in veterinary and human medicine (Wang and Wang 2016, Korzeniewska and Harnisz 2020). Considering their occurrence as demonstrated in various studies, the WWTP effluents containing high concentrations of these antibiotics are discharged into surface water, especially rivers, which subsequently become the main outlets of such antimicrobials into the rest of the natural aqueous environment. Some of the antimicrobial classes detected in the aqueous environments are described briefly.

2.2.1 β-lactams

This group consists of a class of broad-spectrum antimicrobial compounds, which are the most frequently administered antimicrobials in all European countries similar to the rest of the world (Korzeniewska and Harnisz 2020). β-lactams are structurally characterized by a β-lactam ring which is highly susceptible to hydrolysis by a variety of reagents, both biotic (enzymatic and biological degradation) and abiotic (chemical degradation) processes. The β-lactam ring is easily destroyed by extremes in pH, light, heat, solvents like water and methanol (Deshpande et al. 2004). The variation of β-lactams occurrence in the environment during the year depends on therapeutic usage and consumption patterns. They rarely persist in the environment due to their unstable property in spite of being widely consumed. However, the β-lactams, penicillin G and V were mostly found in raw wastewater samples, whereas amoxicillin, a synthetic derivative of penicillin, and cefuroxime, a second-generation cephalosporin, are much more stable and are frequently found in hospital effluents as well as raw wastewater (Michael et al. 2013, Harrabi et al. 2018).

2.2.2 Aminoglycosides

The usage of aminoglycosides in clinical practice is often restricted due to their adverse effects and toxic potential, which makes their contamination of the aqueous environment mostly associated with their application in veterinary medicine. Despite their low consumption, aminoglycosides have been detected in raw and treated wastewater, which was attributed to effluents from hospitals and wastewater from factories producing these pharmaceuticals (Tahrani et al. 2016). Several aminoglycosides were detected in WWTPs influents and effluents in various ranges, including kanamycin B, sisomicin, gentamicin, and neomycin (Tahrani et al. 2016). In Poland for example, the occurrence of aminoglycosides neomycin, streptomycin, and dihydrostreptomycin investigated in water samples drawn from supply systems in different animal farms yielded only neomycin (Gbylik-Sikorska et al. 2015).

2.2.3 Quinolones and fluoroquinolones

The quinolone class of chemically synthesized antibiotics was among the latest to be introduced in clinical practice. They are frequently used and their consumption in human medicine is estimated to account for 7% of the total antimicrobial consumption (Szymańska et al. 2019). Fluoroquinolones are mobile in the aquatic environment due to their hydrophilic property, which explains their presence in both groundwater and drinking water samples (Hanna et al. 2018, Reis et al. 2019). It is this ability to rapidly spread in the environment that necessitated the inclusion of ciprofloxacin in the watch list of the EU commission, Decision of 2018. Their occurrence in different aqueous environmental matrices has been reported, with the maximum concentrations typically occurring in hospital effluents and WWTP influents. Ciprofloxacin and ofloxacin appear to be the dominant ones detected in wastewater with high detection frequency and high concentration (Lindberg et al. 2007). Several other quinolones and fluoroquinolones, which include pipemidic acid, nalidixic acid, moxifloxacin, and gatifloxacin, have been detected in WWTPs (Zhang and Li 2011). European WWTP influents and effluents have reported quinolones in various concentrations (Santos et al. 2013). Ciprofloxacin, for example, has been found in hospital effluent from Spain, Sweden, Portugal, and Italy at concentrations of tens of μg L^{-1} (Lindberg et al. 2004, Gracia-Lor et al. 2012, Verlicchi et al. 2012, Gros et al. 2013, Santos et al. 2013), which presents hospital effluents as important input sources of quinolones into wastewater.

2.2.4 Sulfonamides and trimethoprim

Sulfamethoxazole is the representative drug among the sulfonamides and is currently the most frequently used drug in this class, making the compound one of the most common substances found in the environment (Hanna et al. 2018, Loos et al. 2018). Studies have shown that sulfonamides are partially excreted unchanged, primarily through urine (Prescott 2013). Their occurrence in different aqueous environmental matrices in various regions over the last decades has been documented. The concentration of sulfonamides in WWTP influents and effluents was found to range from tens to hundreds of ng L^{-1}, and this is attributed to their consumption in the community sector (Golovko et al. 2014, Papageorgiou et al. 2016). Sulfamethoxazole, the most common sulfonamide, has been found in WWTP influents and effluents in Germany, Portugal and Kenya (Santos et al. 2013, Rossmann et al. 2014, Ngumba et al. 2016). Very high concentrations of sulfonamides (20×10^3 ng mL^{-1}) have been detected in pig farm wastewater, and the detection of sulfamethazine, for example, has been suggested to serve as a marker for livestock source contamination in Vietnam (Managaki et al. 2007). Trimethoprim is used in combination with sulfonamides to increase the bactericidal effect achieved through synergy. A combination of trimethoprim and the sulfonamide, sulfamethoxazole (Co-trimoxazole), has widespread use in both human and veterinary medicine. Trimethoprim has been determined in WWTPs and hospital effluents in the UK, Croatia, Greece, Italy, and Sweden (Kasprzyk-Hordern et al. 2009, Verlicchi et al. 2012, Santos et al. 2013, Kosma et al. 2014, Mendoza et al. 2015).

2.2.5 Tetracyclines

Tetracyclines comprise both natural antibiotics such as tetracycline, chlortetracycline, oxytetracycline, and semi-synthetic drugs such as doxycycline and demeclocycline. Tetracycline is a broad-spectrum antibiotic that has been widely used to prevent infections in humans and animals, and as a growth promoter in animal feeding at sub-therapeutic dose levels (Sabino et al. 2019). Tetracycline is widely distributed in animal farms, and in the gut of migratory birds, and has potential side effects on human health (Cao et al. 2020). Although they are less frequently used in human medicine, they have been identified in samples of wastewater, surface water, and drinking water (Azanu et al. 2018b, Hanna et al. 2018). Humans and animals excrete over 70% of tetracycline antibiotics in an active form to the environment and, owing to their highly hydrophobic

property and low volatility, tetracyclines are very stable in the aquatic environment and are commonly detected in WWTPs (Daghrir and Drogui 2013). They form stable complexes with cations which makes them more likely to bind to suspended matter or sewage sludge during wastewater treatment (Collado et al. 2014). Tetracycline is the most common substance detected in WWTPs (Opriş et al. 2013, Vergeynst et al. 2015). Five tetracycline antibiotics, including doxycycline, tetracycline, oxytetracycline, and chlortetracycline were found in hospital samples and WWTPs influent and effluent in Sweden, Hong Kong, Norway, and Germany (Yang et al. 2005, Lindberg et al. 2006, Minh et al. 2009, Watkinson et al. 2009, Rossmann et al. 2014).

2.2.6 Macrolides

Macrolide antibiotics are a critical class of compounds due to their significant consumption in hospitals and they enter into wastewater as unchanged parent compounds upon excretion via bile and feces after being hardly metabolized in the body and the continuous application in veterinary and human medicine has contributed to the presence of these antibiotics in aqueous matrices due to their stability (Nnadozie et al. 2017). These compounds are prevalent in the natural environment, especially WWTPs, where the quantities of the macrolides- tylosin, roxithromycin, azithromycin, and clarithromycin have been determined in raw sewage and the treated effluent (Yang and Carlson 2004, Göbel et al. 2005, Petrovic et al. 2006, Spongberg and Witter 2008, Lin et al. 2009, Watkinson et al. 2009). Some macrolides, particularly clarithromycin and azithromycin are among the most commonly detected antimicrobials (Verlicchi et al. 2012, Loos et al. 2018). Wastewater effluents form key input sources of macrolides into rivers as evidenced by their presence in rivers in Spain and France (Valcarcel et al. 2011, Moreno-González et al. 2014). Although erythromycin is the parent antibiotic, a high concentration of its metabolite dehydrated erythromycin-H_2O has been found in both raw sewage and treated wastewater effluent (Kasprzyk-Hordern et al. 2009, Minh et al. 2009). The parent antibiotic, erythromycin was found in both the influent and effluent (Ternes et al. 2007).

The annual consumption of various antibiotic classes among communities and hosptials varies from one region to another with β-lactams being the most widely prescribed compounds due to their wide margin of safety. For instance, the consumption of antibiotics in hospitals located in

the city of Pécs, Hungary, in the years 2006 and 2019 showed considerable variation as demonstrated in **Figures 2**a and b.

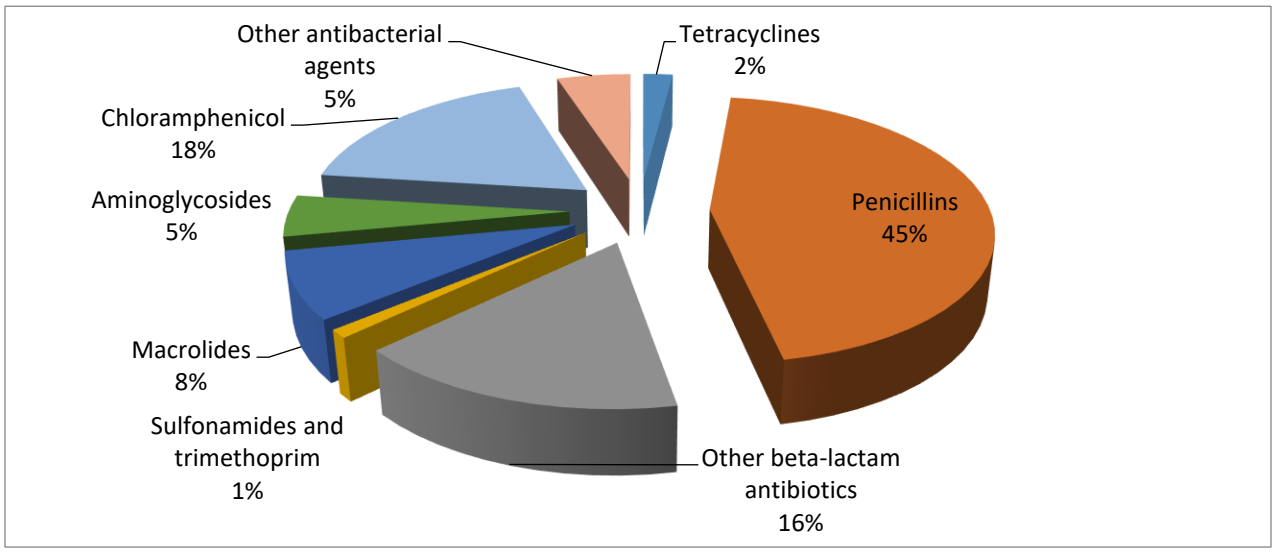


Figure 2a. Antibiotic consumption in hospitals in the city of Pécs, Hungary, in 2006 (DDD - 163.622). Source; PTE Central Clinical Pharmacy data.

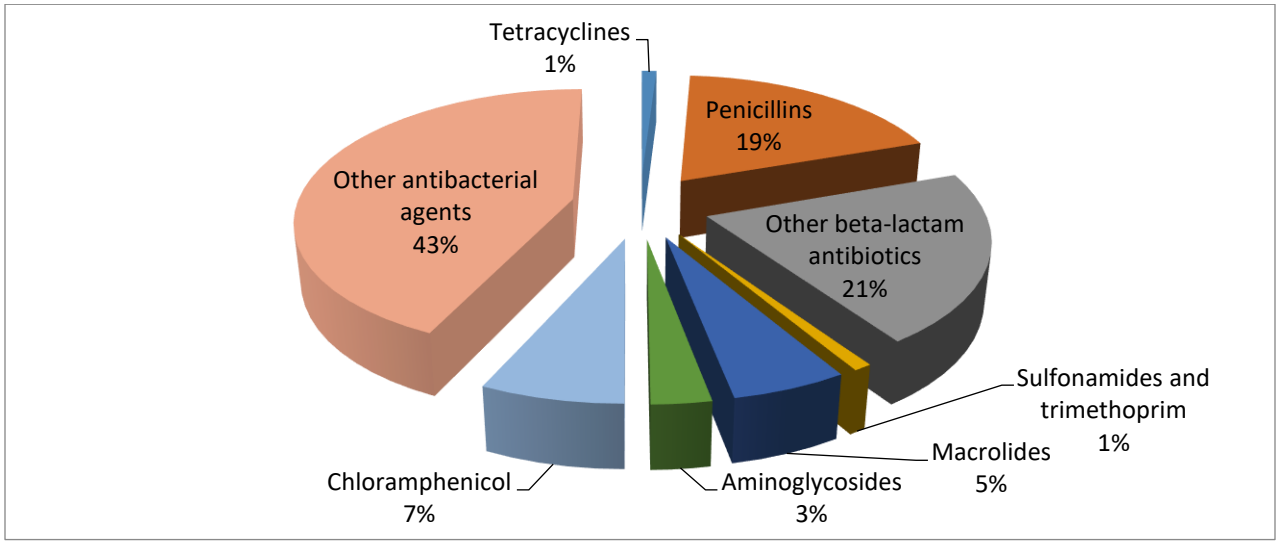


Figure 2b. Antibiotic consumption in hospitals in the city of Pécs, Hungary, in 2019 (DDD-323864.1). Source; PTE Central Clinical Pharmacy data.

The residues of various representative substances of antibiotic classes that have been detected at various concentrations in both raw wastewater (including hospital effluents) and treated wastewater in different regions are shown in **Table 1**.

Table 1. Occurrence of antimicrobial compounds in raw wastewater (hospital effluents and WWTP influent) and treated wastewater in ng/L (data adapted from Tahrani et al 2016, Felis et al 2020).

Class/Compound	Raw wastewater	Treated wastewater
β-lactams Penicillin G	18–6196 (Loos et al. 2013, Ruff et al. 2015, Loos et al. 2018), 13800 (Watkinson et al. 2009)	47–1205 (Loos et al. 2013, Ruff et al. 2015, Loos et al. 2018), 2000 (Watkinson et al. 2009)
Penicillin V	nd-160 (Gros et al. 2013, Michael et al. 2013)	
Amoxicillin	33800 (Azanu et al. 2018b), 2.0-57, hospital effluent (Azanu et al. 2018b, Thai et al. 2018)	nd-116400 (Gros et al. 2013, Azanu et al. 2018b)
Cefotaxime	1100 (Watkinson et al. 2009)	<15 (Watkinson et al. 2009)
Cefuroxime	49-24380 (Ribeiro et al. 2018), 246, hospital effluent (Thai et al. 2018)	7860 pharma factory (Thai et al. 2018)
Aminoglycosides Kanamycin B	500-7500 (Tahrani et al. 2016)	700-5400 (Tahrani et al. 2016)
Sisomicin	2300-6700 (Tahrani et al. 2016)	1000-3900 (Tahrani et al. 2016)
Gentamicin	500-1600 (Tahrani et al. 2016), 400-7600 (Löffler and Ternes 2003)	200-600 (Tahrani et al. 2016)
Neomycin	1800- 16400 (Tahrani et al. 2016)	400-11200 (Tahrani et al. 2016)
Amikacin	2300 (Tahrani et al. 2016)	1000 (Tahrani et al. 2016)
Streptomycin	2700 (Tahrani et al. 2016)	1200 (Tahrani et al. 2016)
Fluoroquinolones Ciprofloxacin	3700 (Verlicchi et al. 2012), 34500 (Matongo et al. 2015), 3600 -101000, hospital effluent (Lindberg et al. 2004) 1400-26000, hospital effluent (Verlicchi et al. 2012)	1100 (Verlicchi et al. 2012)
Levofloxacin		4-836 (Rossmann et al. 2014)
Ofloxacin	11.1-1330 (Birošová et al. 2014, Dong et al. 2016), 23-510 hospital effluent (Verlicchi et al. 2012)	0.3-527 (Golovko et al. 2014, Dong et al. 2016)
Norfloxacin	<LOQ-5411 (Dong et al. 2016, Östman et al. 2017), 450-2200, hospital effluent (Verlicchi et al. 2012)	0.2-628 (He and Blaney 2015, Dong et al. 2016)
Sulfonamides Sulfamethoxazole	6500, 8700, 13000, 2000, 54800 (Lindberg et al. 2004, Verlicchi et al. 2012, Santos et al. 2013, Ngumba et al. 2016)	3340 (Ngumba et al. 2016)

Sulfapyrydyne	60-230 (Göbel et al. 2005)	0.4-230 (Göbel et al. 2005)
Sulfamethazine	4010 (Li and Zhang 2011)	
Trimethoprim	1500- 6000 (Verlicchi et al. 2012, Kosma et al. 2014), 4250-72900 (Ngumba et al. 2016), 100-4300 (Göbel et al. 2005, Watkinson et al. 2009, Li and Zhang 2011), <3000, hospital effluent (Santos et al. 2013)	60–3000 70, 65-800 (Göbel et al. 2005, Li and Zhang 2011, Loos et al. 2018)
Tetracyclines Tetracycline	58-1960 (Azanu et al. 2018b, Lorenzo et al. 2018), 13-1598, hospital effluent (Azanu et al. 2018b, Lorenzo et al. 2018, Wang et al. 2018a)	1400-146000 (Opriş et al. 2013, Vergeynst et al. 2015)
Doxycycline	1.8-264 (Azanu et al. 2018b, Hanna et al. 2018), 24-120, hospital effluent (Azanu et al. 2018b) 75-1487, hospital effluent (Azanu et al. 2018b, Wang et al. 2018a)	2210 (Lindberg et al. 2006),1420(Minh et al. 2009), 14-49(Azanu et al. 2018b)
Oxytetracycline	350 (Watkinson et al. 2009), 43-233 (Azanu et al. 2018a), 24-120 hospital effluent (Azanu et al. 2018b)	250 (Watkinson et al. 2009), 2.4-24(Azanu et al. 2018a)
Chlortetracycline	270(Yang et al. 2005)	
Macrolides Erythromycin	830 (Ternes et al. 2007), 1100 (Matongo et al. 2015), 9-294 (Tylová et al. 2013)	620 (Ternes et al. 2007), 160 (Matongo et al. 2015), 886 (Gracia-Lor et al. 2012, Tylová et al. 2013)
Tylosin	1150 (Yang and Carlson 2004), 55-180 (Watkinson et al. 2007)	3400 (Watkinson et al. 2009)
Roxithromycin	810 (Göbel et al. 2005)	540 (Göbel et al. 2005)
Azithromycin	450 (Petrovic et al. 2006), 1083 (Lara-Martín et al. 2014)	400 (Göbel et al. 2005), 0-380 (Al Aukidy et al. 2012, Lara-Martín et al. 2014)
Clarithromycin	1433 (Lin et al. 2009), 122 (Watkinson et al. 2009, Lara-Martín et al. 2014)	996 (Spongberg and Witter 2008), 8-460 (Al Aukidy et al. 2012, Gracia-Lor et al. 2012, Lara-Martín et al. 2014)

2.3 Persistence of antimicrobial pharmaceuticals

The persistence of antimicrobials in water is defined based on their half-life value. For example, according to European legislation on Registration, Evaluation and Authorisation of Chemicals, a chemical is persistent if its half-life in marine water is more than 60 days and 40 days in fresh or

estuarine water. It is considered very persistent when the half-life in marine, estuarine or fresh water is higher than 60 days (Goldenman et al. 2017). The half-life values for the substances selected from the discussed classes of the antimicrobial pharmaceuticals are given in **Table 2**. The given half-life values refer to surface water. These values can be much higher (longer half-life) in the case of groundwater or soil/sediments because of the scarcity or lack of sunlight and aerobic conditions.

Table 2. Half-life of selected antimicrobials in surface water (data adapted from Felis et al 2020).

Compound	Surface water half-life
Amoxicillin	< 1 day
Azithromycin	< 5 h
Tylosin	9.5-54 days
Erythromycin	< 17 days
Ciprofloxacin	< 46 h
Levofloxacin	6.3 days
Ofloxacin	10.6 days
Norfloxacin	77 days
Sulfamethoxazole	< 20.3 days
Sulfamethazine	< 4.2 days
Trimethoprim	11.8 days

2.4 Antimicrobial pharmaceuticals promote the evolution and transmission of resistance

Antibiotics overuse, inappropriate prescription, and their extensive use in agriculture are linked to the widespread occurrence of antibiotics in the environment (Chowdhury et al. 2017). These and other anthropogenic activities that result in the discharge of wastewater containing antibiotics and/or their metabolites into environmental matrices have been attributed to the increasing antimicrobial resistance due to the rapid evolution of bacteria facilitated by the acquisition of resistance from the reservoir of ARGs, which has a direct impact on the control of microbial pathogens in humans and animals *(*Kemper 2008, Zhang et al. 2009b*)*. Aquatic environments,

especially WWTPs, serve as sinks for massive loads of pharmaceutical compounds, including personal care products and antibiotics, and provide optimal conditions where antibiotic resistant bacteria develop and proliferate and ARGs spread (Kim et al. 2007). Dissemination of antibiotics and ARGs occurs in habitats that provide ideal environments for their spread and circulation between humans, animals, and the external environment. **Figure 3** depicts several habitats that are ideal for recombination events and subsequent genetic exchange where the future evolution of resistance among microbes in the environment occurs. Human and animal microbiota consisting of diverse bacterial species form the primary habitat in which antibiotics assigned for prevention or therapy exert their actions. Animal and human digestive systems provide suitable residence for bacteria along with sub-lethal doses of antibiotics, which might be potential niches for the propagation of antibiotic resistance (Chopra and Roberts 2001). Environments where susceptible individuals are often overcrowded with possible exposure to bacterial genetic exchange, such as hospitals, nursing/retirement homes (which serve as long-term care facilities-LTCF), and animal farms, constitute the secondary habitat. Antibiotics and other antimicrobial residues in wastewater that originate from secondary residences find their way into WWTPs where they mingle with bacteria. The wastewater treatment facilities, which constitute the tertiary habitat, provide suitable conditions for mixing and genetic exchange (Berendonk et al. 2015). Soil or sediments and surface or groundwater environments provide the final habitat in which bacteria originating from previous habitats continuously mix and interact with the broader microbial communities in the environment. The interconnection among these habitats creates a niche that breeds resistant bacteria and ARGs, which circulate in the ecosystem and may eventually be re-introduced into human and animal environments. The strategies employed by humans to regulate the introduction of active antimicrobial agents and bacteria into these sites, such as pre-treatment of hospital effluents and enhancing antibiotic stewardship programs, minimize the possibility of the microbes evolving antibiotic resistance.

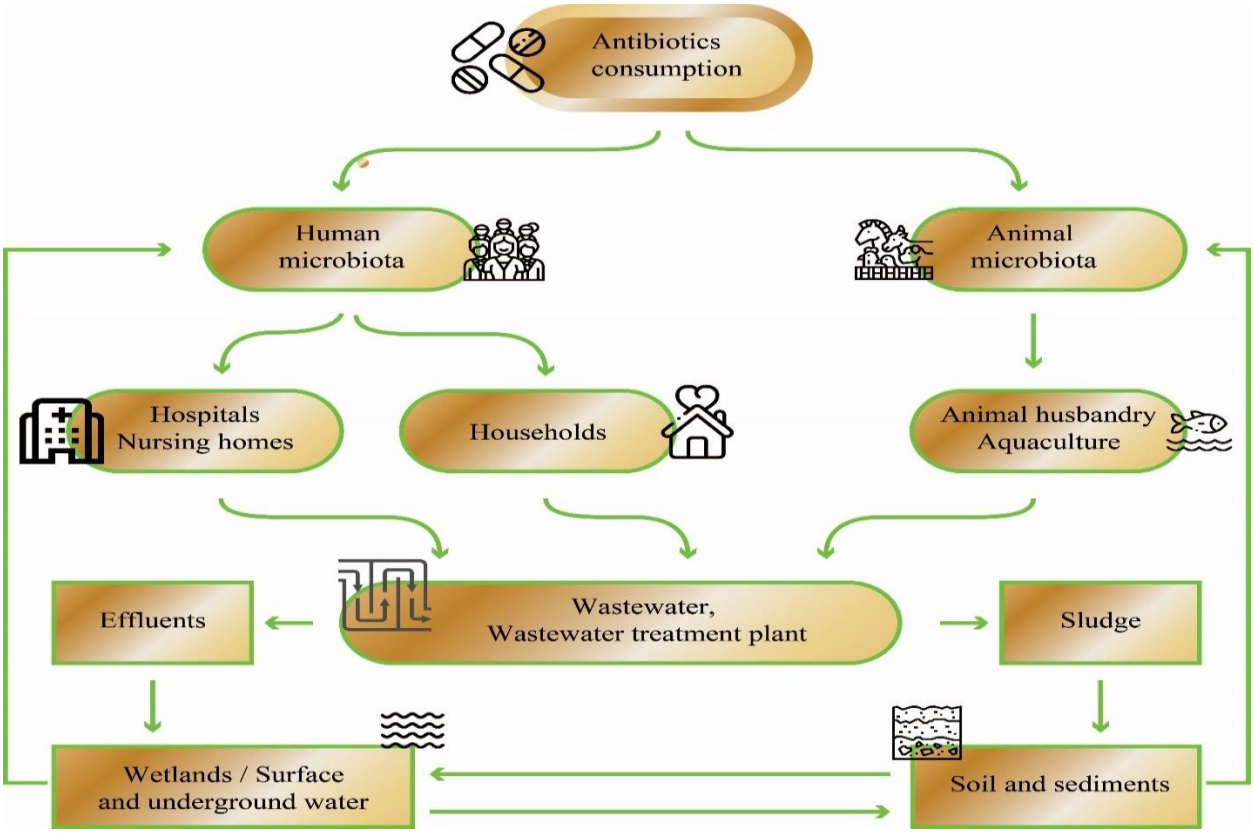


Figure 3. Ideal habitats for recombination events and subsequent genetic exchange where the future evolution of resistance among microbes in the environment occurs.

Antibiotics and bacteria from the human population, veterinary medicine, and food-producing animals taking antibiotics enter various habitats such as soil and surface water via excreta, through effluents, and biosolids from wastewater treatment plants. Antibiotics, ARGs, and resident environmental bacteria mix in the various compartments, spurring the emergence and spread of ARB and ARGs in the bacterial community, and they can eventually end up in animal hosts, including humans.

Significant genetic variation is associated with mechanisms of genetic exchange occurring frequently among microbial populations and communities spurred by habitats that provide suitable biological interconnection, generate variation, and offer chances for specific selection, leading to the genetic evolution of resistant bacteria (Baquero et al. 2008). Mutation is a key event that can form the basis for the selection of resistance in the mix of bacteria and antimicrobial compounds in the various habitats. Mutations drive antibiotic resistance by occurring spontaneously in the

bacterial genome, and the mutants propagate the resistance to the subsequent progeny through vertical evolution and natural selection created by antibiotic pressure (Baquero et al. 2008). **Figure 4** illustrates the role of antibiotics in the selection and proliferation of resistant bacteria driven by mutation.

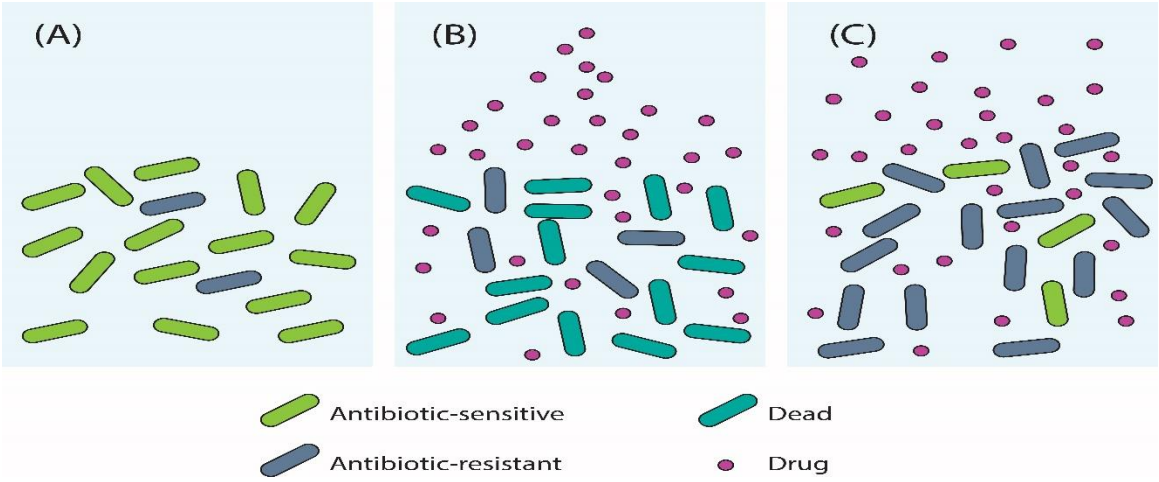


Figure 4. Resistance evolution driven by the presence of antibiotics (Figure adapted from Mutuku et al 2022).

a. Mutant bacteria occur frequently in large population sizes. The frequency of mutants is low in the absence of antibiotics since resistance typically imposes a fitness cost, **b.** Resistant bacteria divide faster than sensitive bacteria in an environment created by the presence of antibiotics. **c**. Resistant bacteria finally dominate the population, and the antibiotic becomes ineffective.

Since DNA replication is not perfect, cell division may result in random changes to the DNA sequences of descendent cells. The biological effects of the resultant mutations on the cells that carry them can range from insignificant to disastrous. Some mutations, for example, alter the cellular proteins that are frequently targeted in antibiotic treatment. A random mutation that alters a cellular protein required for a specific antibiotic to enter the cells of its target bacterial species blocks the antibiotic entry into the mutant cell and interferes with protein synthesis. Unlike in the absence of antibiotics in which an antibiotic resistance mutation does not provide a selective advantage to a cell, in the presence of antibiotics, the mutant reproduces normally. In the presence of the antibiotics, wild-type drug-sensitive cells would either fail to reproduce or die (Genereux and Bergstrom 2005). Typically, antibiotics designed to kill bacteria end up selecting for bacteria

that do not respond to the antibiotics. Antibiotic resistance can also be driven by horizontal evolution through gene exchange mechanisms occurring in intra and inter-species (Touchon et al. 2017). Conditions within the environment, especially the WWTPs provide cell proximity, which favors horizontal evolution (or lateral gene transfer). Horizontal gene transfer (HGT) follows either or a combination of the three routes (conjugation, transformation, and transduction) where genetic material is obtained from antibiotic resistant bacteria in each case, and the recipients become resistant (Von Wintersdorff et al. 2016). Lateral transfer of genetic material occurs frequently among bacterial populations aided by resistance plasmids (R-plasmids), which contain antibiotic resistance genes and have been linked to global antibiotic resistance spread in the vast majority of Gram-negative bacteria (Berglund 2015). **Figure 5** illustrates the mechanisms of horizontal gene transfer in bacteria.

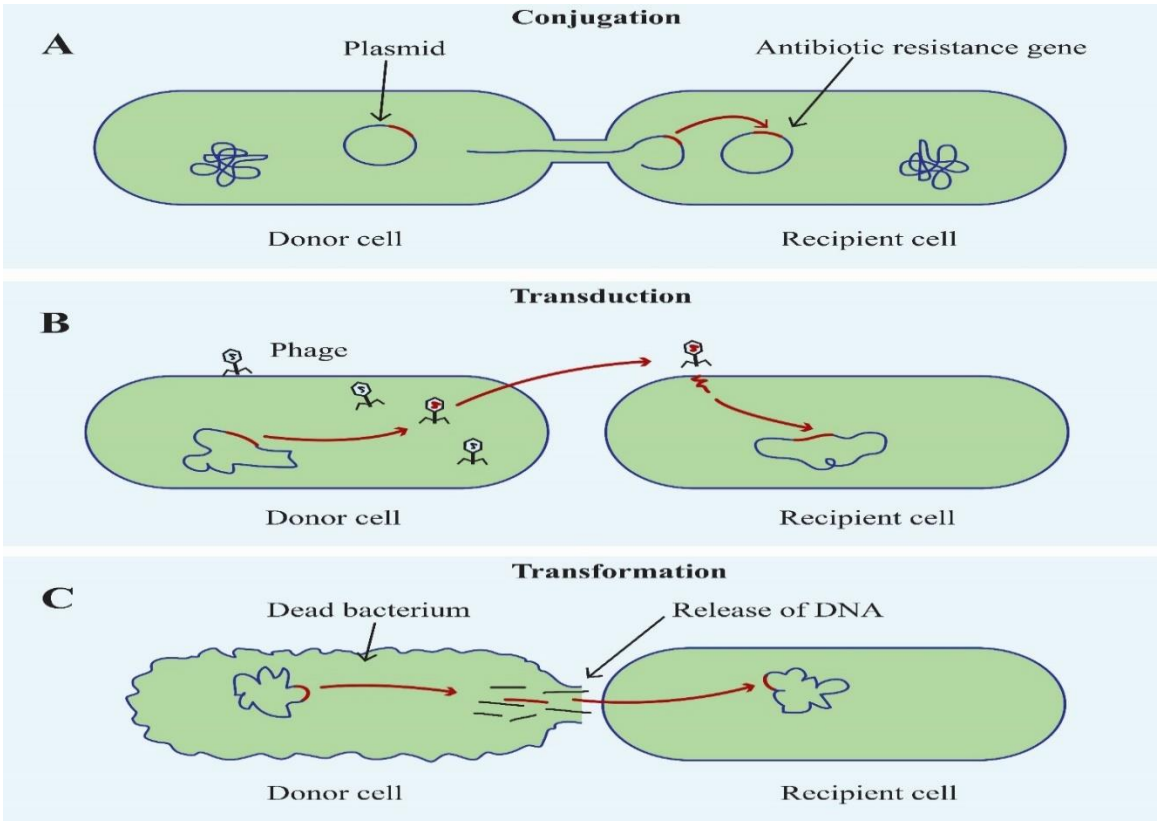


Figure 5. Mechanisms of horizontal gene transfer where bacterial DNA can be transferred from one bacterium to another (Figure adapted from Pang et al 2019).

(A) Conjugation involves direct contact transfer of mobile plasmids between the donor cell and the recipient cell (B) Transduction refers to the transfer of DNA from one bacterium to another mediated by bacteriophages (C) In transformation bacteria pick up free fragments of DNA from the environment and integrate them into their genome.

2.5 β-lactam antibiotics structure and mechanism of action

β-lactam antibiotic group is a class of broad-spectrum pharmaceutical antimicrobials that include all agents whose molecular structure consists of a β-lactam ring. Penicillin derivatives (penams), cephalosporins (cephems), carbapenems, and monobactams form this group (Holten and Onusko 2000). Together with other antibiotics such as vancomycin, bacitracin, fosfomycin, and isoniazid, they inhibit bacterial cell wall synthesis. β-lactams are the most frequently administered antimicrobials across the world (Korzeniewska and Harnisz 2020). They are structurally characterized by a β-lactam ring which is highly susceptible to hydrolysis by a variety of reagents (Deshpande et al. 2004). **Figure 6** shows the chemical structures of β-lactams and **Figure 7** shows the generalized classification of β-lactams antibiotics and other cell wall inhibitors.

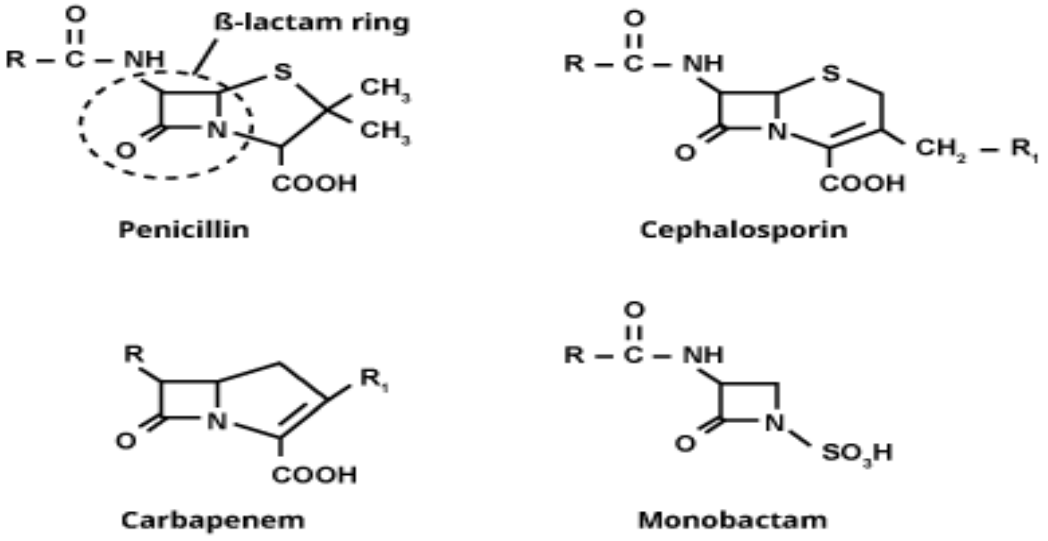


Figure 6. Chemical structures of β-lactam antibiotics showing the common β-lactam ring.

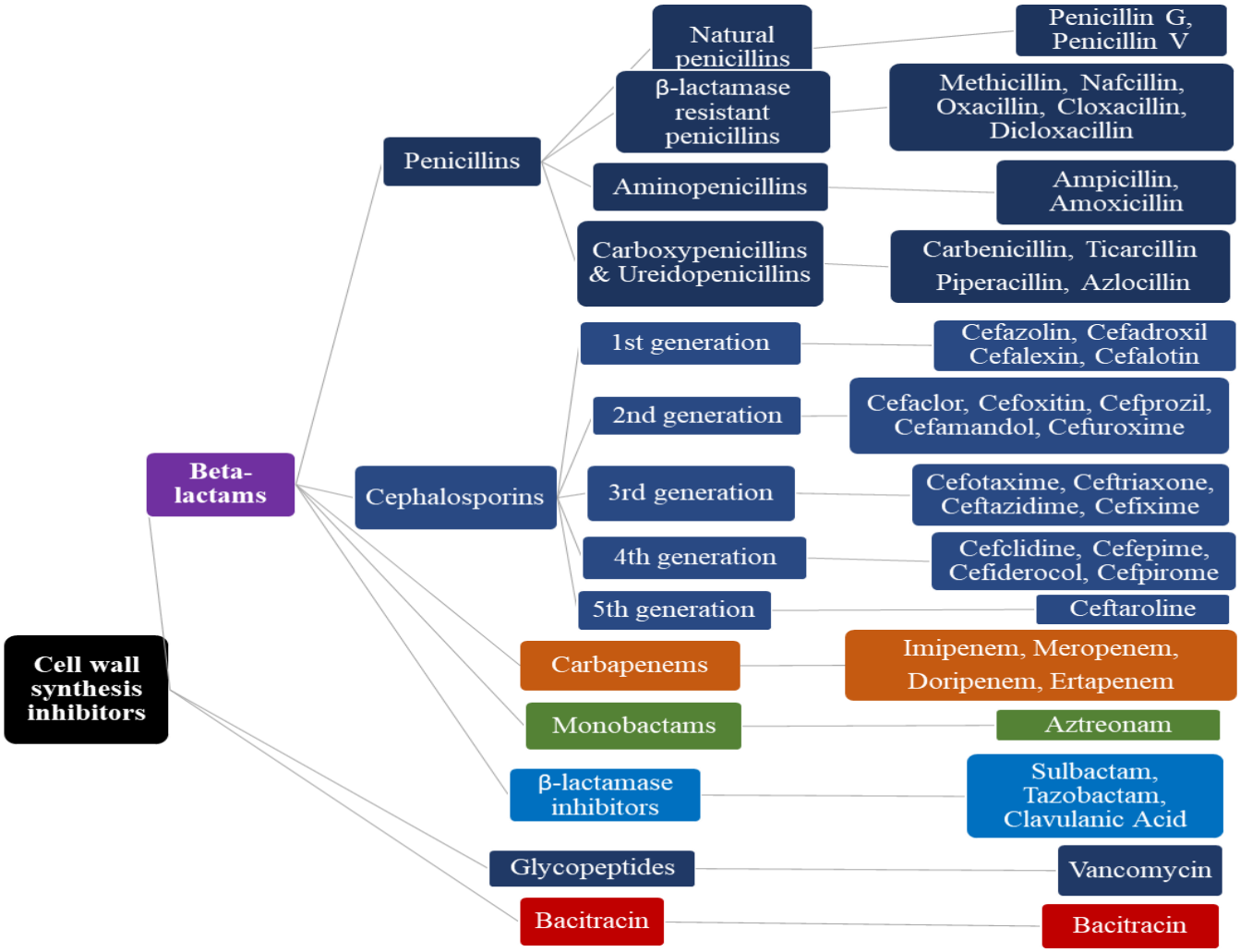


Figure 7. An illustration of a generalized classification of different groups of β-lactam antibiotics and other cell wall synthesis inhibitors.

All β-lactam compounds interfere with the synthesis of the bacterial cell wall, which is made up of extensively cross-linked peptide and glycan chains that give the cell structure stability and rigidity. These chains are made up of two amino sugars called N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG). The peptidoglycan molecule is formed by linking a pentapeptide side chain to NAM sugar. The cleavage of terminal D-alanine of the peptide chains by transpeptidases catalyzed by penicillin-binding proteins (PBP), which are transmembrane surface enzymes found in bacteria, is the final step in its synthesis. Beta-lactams inhibit cell wall synthesis by irreversibly binding to the penicillin-binding proteins required for the final crosslinking (transpeptidation) step in the synthesis of peptidoglycan for cell wall construction. Inhibition of

this catalyzed process of transpeptidation results in a loosely knit structure of the cell wall. Cell wall deficient organisms result when susceptible bacteria multiply in the presence of a β-lactam compound. The interior of the bacterium is hyperosmotic which results in osmotic drive and eventual cell lysis. The most clinically important are the third-generation cephalosporins, 'anti-pseudomonal' β-lactam/β-lactamase inhibitor combinations, and carbapenems (Partridge et al. 2009a*)*. Being the most widely used antibiotics, β-lactams resistance is a severe threat because they have low toxicity and are used to treat a broad range of infections. **Figure 8** shows a cross-linked peptidoglycan structure.

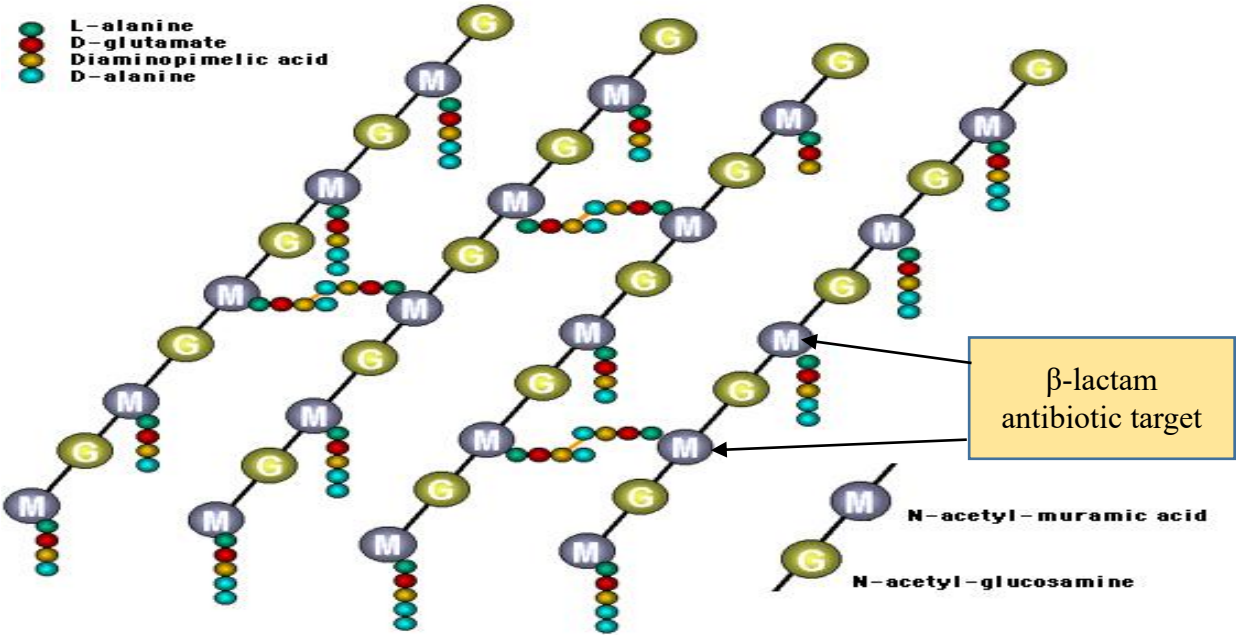


Figure 8. Mechanism of action of β-lactam antibiotics showing targeted NAM molecules, which interfere with transpeptidation (Figure adapted from Talaro 2017).

2.6 Mechanisms of resistance to β-lactams in Enterobacterales

The mechanisms of β-lactam resistance include inaccessibility of the antibiotics to their target enzymes, modification of target enzymes, and direct deactivation of the antibiotics by β-lactamases (Tang et al. 2014). Among the members of Enterobacterales, β-lactam resistance occurs commonly through two mechanisms.

2.6.1 Porin defects

The outer membrane proteins (OMPs) of Gram-negative bacteria form water-filled pores through which hydrophilic substances, including β-lactams, can passively cross the outer membrane into the periplasm. Loss, reduced expression, or reduced permeability of porins resulting from mutations are alone, not sufficient to result in clinically-significant resistance, but there are examples of β-lactams resistance due to a CTX-M or AmpC β-lactamases and carbapenemases (e.g., IMP and KPC enzymes) plus a porin defect (Wang et al. 2009). This form of β-lactam resistance appears particularly important in *K. pneumoniae*. The main porins in *K. pneumoniae* are OmpK35 and OmpK36 and whereas OmpK35 defects are common in isolates expressing ESBL, additional defects in OmpK36 are associated with carbapenem resistance (Martínez-Martínez 2008).

2.6.2 Enzymatic inactivation

The key resistance mechanism to β-lactam antibiotics in the Enterobacterales is the expression of β-lactamase enzymes encoded by *bla* genes either on a plasmid or chromosomal DNA (Deshpande et al. 2004) that hydrolyze the β-lactam ring, resulting in inactivation or degradation. *bla* genes associated with mobile genetic elements often coexist with other antimicrobial resistance determinants, increasing the possibility of multidrug resistance and dissemination (Tennstedt et al. 2003, Schluter et al. 2007). Many different types of β-lactamases can confer resistance to each of the most clinically-important β-lactam types and a single amino acid difference may affect the phenotype conferred. β-lactamases can be categorized (1) into classes A–D based on Ambler molecular classification or (2) according to the Bush-Jacoby (functional) grouping (Ambler 1980, Bush and Jacoby 2010a), which encompasses ESBL, AmpC cephalosporinases, and metallo-β-lactamases. Microbial resistance through ESBL has been widely reported in Europe and in the United States since the introduction of third-generation cephalosporins in clinical practice (Livermore and Hawkey 2005). ESBL are a group of enzymes capable of hydrolyzing a variety of β-lactams, including cephalosporins such as ceftazidime, cefotaxime, ceftriaxone, cefepime, and monobactams like aztreonam in addition to penicillin's but do not hydrolyze cephamycins like

cefoxitin and carbapenems (Bradford 2001). Earlier studies reported *Klebsiella* species and *Escherichia coli* as the main ESBLs producers but they can be widely detected in other Gram-negative bacteria such as *Enterobacter*, *Salmonella*, *Citrobacter*, *Serratia marcescens*, *Proteus* species, and *P. aeruginosa* (Lautenbach et al. 2001).

2.7 Classification of extended spectrum β-lactamases (ESBL)

There are two most accepted methods of classification for ESBLs.

Ambler molecular classification scheme: This scheme places β-lactamases into four major groups (A, B, C, and D). This classification system considers protein homology, or sequence similarity, as opposed to phenotypic characteristics. The β-lactamases of classes A, C, and D are categorized as serine β-lactamases in the Ambler classification scheme, while the enzymes of class B are placed under metallo-β-lactamases. However, most of the ESBLs are of molecular class A, with the exception of OXA-type enzymes (which are class D enzymes) (Paterson and Bonomo 2005).

Bush-Jacoby-Medeiros (traditional) or functional classification scheme: Based on the functional similarities (substrate and inhibitor profile), there are four main groups and multiple subgroups in this system (Rasmussen and Bush 1997).

Group 1 Cephalosporinases: This family is a member of the molecular class C, which is encoded on the chromosomes of Enterobacterales and a few other microbes. They appear to be more active against cephalosporins than benzylpenicillin. β-lactamase inhibitors (BLI) such as clavulanic acid, sulbactam, and tazobactam do not inhibit cephalosporinase activity but are active on cephamycins such as cefoxitin. (Gurung et al. 2010).

Group 2 serine β-lactamases: This is the largest category of β-lactamases, and corresponds to molecular classes A and D, which reflect the initial TEM and SHV genes. This group involves penicillinases and cephalosporinases. Moreover, these enzymes are inhibited at various degrees by the β-lactamase inhibitors, clavulanic acid, and tazobactam (Picão et al. 2009). Furthermore, β-lactamase inhibitor proteins inhibit many class A TEM β-lactamases (Jacoby 2009).

Group 3 Metallo-β-lactamases (MBLs): A distinct class of β-lactamases in both structural and functional forms. Their main structural difference from the other β-lactamases is the presence of a zinc ion at the active site. They were distinguished functionally primarily by their ability to hydrolyze carbapenems; however, some serine β-lactamases are reported to have acquired this

ability as well. Metallo-β-lactamases (MBLs) have a lower affinity or hydrolytic capability for monobactams than serine β-lactamases and are not inhibited by tazobactam or clavulanic acid. Metal ion chelators such as EDTA, on the other hand, inhibit them (Bonnet 2004).

Group 4 β-lactamases: This category contains enzymes that have not been fully characterized and classified and may fall into one of the existing enzyme groups. Moreover, this group contains penicillinases that are not inhibited by clavulanic acid, and do not belong to any of the corresponding molecular classes (Marchiaro et al. 2008).

2.8 Diversity of extended spectrum β-lactamases

TEM β-lactamases (class A): The TEM-type β-lactamases are most often found in *E. coli* and *K. pneumonia*e, but also occur in other species of Gram-negative bacteria with increasing frequency. The TEM types of ESBL are derived from TEM-1 and TEM-2, in which TEM-1 was first reported in 1965 from an *E. coli* isolate from a patient in Athens, Greece (Bradford 2001). An earlier report states that TEM-1 confers about 90% resistance to ampicillin and about 140 TEM types have been described (Datta and Kontomichalou 1965).

SHV β-lactamases (class A): SHV-1, which is mostly harbored by *K. pneumoniae* and shares 68% structural and sequence similarity with TEM-1, is another popular type of β-lactamase. SHV-1 accounts for up to 20% of this species' plasmid-mediated ampicillin resistance. Furthermore, although SHV-5 and SHV-12 are the most frequent, more than 60 SHV variants have been described (Jacoby and Munoz-Price 2005).

CTX-M β-lactamases (class A): In comparison to other oxyimino-β-lactam substrates such as ceftazidime, ceftriaxone, or cefepime, this enzyme is more effective against cefotaxime. They are examples of β-lactamase genes that are plasmid-acquired, and are usually found on the chromosome of *Kluyvera* species, which are rare pathogenic commensal organisms. CTX-M shares 40% sequence similarity with the TEM or SHV β-lactamases. Studies show that CTX-M enzymes contain more than 80 variants, most of which have been characterized. Evidently, they have mostly been found in the strains of *Salmonella enterica* „serovar", *Typhimurium,* and *E. coli*, but have also been described in other species of Enterobacterales (Sykes 1982).

OXA β-lactamases (class D): The OXA-type enzymes are a growing family of ESBLs that are completely different from the TEM and SHV enzymes, with only 20% sequence similarity. They

are members of the molecular class D and the functional group 2d. Resistance to the antibiotics ampicillin and cephalothin is also conferred by these OXA enzymes. They are also distinguished by their high hydrolytic activity against oxacillin and cloxacillin, as well as their resistance to clavulanic acid (Rasmussen and Bush 1997).

2.9 Resistance to β–lactams in *Pseudomonas aeruginosa*

Among the β-lactam antibiotics group, piperacillin and ticarcillin (penicillins), ceftazidime (3rd. generation cephalosporin), cefepime (4th. generation cephalosporin), aztreonam (monobactam), imipenem, meropenem, and doripenem (carbapenems) are most effective and commonly used in the control of *P. aeruginosa (*Köck et al. 2010*).* Resistance in *P. aeruginosa* to β-lactams may be due to a plethora of different resistance mechanisms including, chromosomal hyperproduction of ampicillin C-type (AmpC) β-lactamases, acquired cephalosporinases or carbapenemases, overexpression of efflux pumps, modifications in the PBPs, and downregulation or absence of the OprD porin proteins (i.e., porin mutants) (Khuntayaporn et al. 2013).

AmpC β-lactamase (Cephalosporinases): The production of endogenous β-lactamase such as AmpC β-lactamase (chromosomal cephalosporinase) in *P. aeruginosa* can be induced by several β-lactams including, benzylpenicillin, narrow-spectrum cephalosporins, and imipenem (Yamane et al. 2004). *P. aeruginosa* is naturally susceptible to carboxypenicillins, ceftazidime, and aztreonam but it can acquire resistance through a gene mutation, which leads to hyper production of AmpC β-lactamase (Poole 2011). The bacterium produces an inducible chromosome encoded AmpC β-lactamase (cephalosporinase) encoded by the ampC gene (Hooper and Gordon 2001) that belongs to molecular class C. The enzyme is usually produced in low quantities ('low-level' expression) and determines resistance to aminopenicillins (ampicillin and amoxicillin) and most of the early cephalosporins. However, chromosomal cephalosporinase production in *P. aeruginosa* may increase from 100 to 1000 times in the presence of inducing β-lactam (especially imipenem) (Yamane et al. 2004).

Class A serine β-lactamases: Various Class A serine extended-spectrum β-lactamases have been described in *P. aeruginosa,* including PER, VEB, GES, and BEL types (Strateva and Yordanov 2009). In addition, ESBL Enterobacterales types of enzymes such as TEM, SHV, and CTX-M have been identified in *P. aeruginosa*, more likely following horizontal gene transfer (Chanawong et al. 2001).

2.10 Carbapenemases

Carbapenems including imipenem, meropenem, ertapenem, and doripenem have been regarded as the mainstay and most potent β-lactams against Gram-negative bacilli due to their high affinity with penicillin-binding proteins, stability against extended-spectrum β-lactamases, and permeability of bacterial outer membranes (Zavascki et al. 2010). Carbapenem hydrolyzing enzymes (carbapenemases**)** have however been detected in the members of Enterobacterales. The majority of these are Class B metallo- β-lactamases, which are dependent on zinc ions for activity rather than the active-site serine found in classes A, C, and D, and confer resistance to carbapenem antibiotics, usually in addition to other β-lactams except aztreonam, and to clinical β-lactamase inhibitors (Cornaglia et al. 2011). The VIM (Verona integron encoded metallo-β-lactamase) enzymes especially *bla*$_{VIM-1}$ and its variants are common in Enterobacterales. Although IMP enzymes have been identified mainly in *P. aeruginosa*, some variants are also common in Enterobacterales. The NDM (New Delhi Metallo-β-lactamase) enzyme first reported in 2009 received a lot of international attention and 15 minor variants have now been identified in a variety of plasmids, strain types, and species (Johnson and Woodford 2013). Certain class A enzymes are categorized as carbapenemases. The most prominent being KPC (*Klebsiella pneumoniae* carbapenemase), which was first identified in the USA in 1996 and is most commonly associated with *K. pneumoniae*, often multi-locus sequence type (ST) 258, (Woodford et al. 2011) but also found in *E. coli*, *Enterobacter* and other species (Nordmann et al. 2009). Carbapenemase activity is also associated with some of the known GES variants especially GES-2 and GES-5, which are associated with clinically-significant resistance to carbapenems (Frase et al. 2009). Class D enzymes (named OXA, for 'oxacillinase'), especially OXA-48 and variants and the related but distinct OXA-181, which can hydrolyze carbapenems and confer low-level resistance to these antibiotics have been extensively reported in Enterobacterales in some locations (Poirel et al. 2012). Similarly, carbapenemases have been identified in *P. aeruginosa* strains. Carbapenemases belonging to the Class A KPC or GES-2 types and MBL of Class B have been described in *P. aeruginosa* (Nordmann Guibert 1998, Lister et al. 2009). GES-2 is a carbapenemase derived from the ESBL GES-1 by point mutation whereas the KPC carbapenemase has been acquired by *P. aeruginosa* through horizontal transfer from Enterobacterales (Weldhagen and Prinsloo 2004,

Villegas et al. 2007). However, the main type of carbapenemases found in *P. aeruginosa* belong to the MBL, and consist of five types: IMP, VIM, NDM, SPM, and GIM (Nordmann and Poirel 2002, Walsh et al. 2005). The encoding genes are mostly harbored on mobile genetic elements, including plasmids, integrons, and cassettes that rapidly favor their dissemination (Strateva and Yordanov 2009).

2.11 Resistance to other antimicrobial agents

2.11.1 Aminoglycosides

Aminoglycosides bind to the aminoacyl-tRNA recognition site (A-site), the decoding centre on the 16S rRNA of the ribosome, thus inhibiting protein synthesis. The most clinically relevant members commonly used against infections caused by Gram-negative bacteria are gentamicin (GEN), amikacin (AMK) and tobramycin (TOB) (Bartlett 2005, Partridge 2015). Resistance development associated with their use is due to acquired inactivation enzymes and 16S rRNA methylases (Poirel et al. 2018).

Aminoglycoside modifying enzymes (AME): These enzymes, mostly linked to genes encoded on mobile elements mainly on integrons, confer resistance to aminoglycosides by acetylating, adenylylating or phosphorylating the aminoglycosides (Ramirez and Tolmasky 2010). The aminoglycoside acetyltransferases act by catalyzing the addition of an acetyl group (CH_3CO) from acetyl coenzyme A to an amine group ($–NH_2$) at positions 1, 2, 3, or 6 of the aminoglycoside structure, which determines the subset of the enzyme (Dolejska et al. 2013). In Gram-negative bacteria, the most common aminoglycosides nucleotidyltransferases are ANT(2″) and ANT(3″) encoded by the genes *aadB* and *aadA*, respectively, both of which can be part of gene cassettes carried in class 1 integrons while streptomycin resistance is mediated by APH(6)-Ia and APH(6)-Id aminoglycoside phosphotransferases encoded by the *strA* and *strB* genes respectively (Ramirez and Tolmasky 2010). More than 50 genes encoding AME have been described, but many variants of the gene cassette-borne acetyltransferases (AAC) appear to dominate in clinically important Gram-negative bacteria (Partridge et al. 2009b). Some mutations of acetyltransferases lead to an ability to acetylate fluoroquinolones. Genes for AME typically occur on integrons with other resistance genes (Ramirez and Tolmasky 2010), which makes AME-harboring isolates multidrug-

resistant. Additionally, aminoglycoside resistance independent of inactivating enzymes is linked to efflux mediated by the MexXY-OprM multidrug efflux system in *P. aeruginosa* (Islam et al. 2009).

16S rRNA methyltransferases (RMTases): These are mainly plasmid-borne 16S rRNA methyltransferases (RMTases), which promote target protection by methylating the 16S rRNA of the 30S ribosomal subunit at the A site that interferes with aminoglycoside binding and results in high-level resistance to aminoglycosides (Wachino and Arakawa 2012). Various 16S rRNA methylases originating from natural aminoglycoside producers as self-protection against these antimicrobials such as ArmA, RmtA/B/C/D/E/F/G/H, and NmpA, have been identified in Gram-negative bacteria, including *Acinetobacter baumannii*, Enterobacterales and *P. aeruginosa* isolates (Yu et al. 2007, Batah et al. 2015). ArmA, RmtB RmtC and RmtF are the most commonly identified enzymes in Enterobacterales, while ArmA, RmtA, RmtB and RmtD are the 16S rRNA methylases promoting aminoglycoside resistance described in *P. aeruginosa* (Jin et al. 2009, Lincopan et al. 2010, Zhou et al. 2010). **Table 3** shows the genes which confer resistance to clinically important aminoglycosides in Enterobacterales (Partridge 2015).

Table 3. Main mobile genes conferring resistance to clinically-important aminoglycosides in Enterobacterales

Mechanism	Resistance to	Mobile elememt
Acetyltransferases		
aacA/aac(6')-Ib	AMK, TOB	Gene cassette
aacA/aac(6')-IIa	GEN, TOB	Gene cassette
aacC/aac(3)-Ia	GEN	Gene cassette
aac(3)-II	GEN, TOB	IS26
Adenylytransferases		
aadB/ant(2")-Ia	GEN, TOB	Gene cassette
Phosphorylases		
aphA6	AMK	ISAba14
aphA15	AMK	Gene cassette
16S rRNA methylases		
armA	AMK, GEN, TOB	ISCR1
rmtB	AMK, GEN, TOB	ISCR3
rmtC	AMK, GEN, TOB	ISEcp1
rmtF	AMK, GEN, TOB	ISCR3-like

AMK, amikacin; GEN, gentamicin; TOB, tobramycin

2.11.2 Quinolones and fluoroquinolones

Quinolones and fluoroquinolones act on DNA gyrase and topoisomerase IV enzymes, which have essential roles during DNA replication. While DNA gyrase introduces negative supercoils, topoisomerase IV removes knots in DNA. The enzymes consist of a tetramer with DNA gyrase having two GyrA plus two GyrB subunits and topoisomerase IV consisting of two ParC plus two ParE subunits. The enzymes introduce double-stranded breaks in the DNA, then re-ligate. Quinolones bind to the cleaved-ligated active site, thereby intercalating into the DNA and blocking the ligation process, resulting in DNA fragmentation, which impairs the function of the two enzymes (Aldred et al. 2014).

Chromosomal quinolone resistance by target site mutations: Mutations usually occur in the gyrase gene, the preferred target of quinolones in Gram-negative bacteria, while additional mutations in the topoisomerase IV gene in some highly resistant isolates have been described (Jacoby 2005). These mutations are found in the "quinolone resistance determining region" (QRDR) of *gyrA* and/or *parC* (Drlica et al. 2009). Mutations in *gyrB* and *parE* are not common, but multiple mutations in *gyrA* and/ or *parC* have been described in highly resistant isolates of fluoroquinolone-resistant *P. aeruginosa* (Muramatsu et al. 2005, Rejiba et al. 2008). Mutations in the DNA gyrase and topoisomerase IV enzymes conferring high-level resistance to fluoroquinolones, especially ciprofloxacin, have also been observed in Enterobacterales (Drlica et al. 2009, Tam et al. 2010). Resistance to fluoroquinolones particularly, ciprofloxacin which is most commonly used against *P. aeruginosa* infections, especially high-level resistance, is predominantly mediated by mutations in the DNA gyrase and topoisomerase IV enzymes similar to Enterobacterales. However, in *P. aeruginosa,* efflux is also a significant contributing factor often in combination with target site mutations (Drlica et al. 2009, Tam et al. 2010). Four members of the resistance nodulation division family of multidrug efflux systems, MexAB-OprM, MexCD-OprJ, MexEF-oprN, and MexXY-OprM are known to accommodate fluoroquinolones and these efflux systems have been implicated in fluoroquinolone resistance in clinical isolates of *P. aeruginosa* (Poole 2000, Reinhardt et al. 2007).

Plasmid-mediated quinolone resistance: Several *qnr* genes, including A, B, C, D, S, and VC families that occur on plasmids, encode proteins that prevent quinolones from entering cleavage complexes by binding to DNA, which decreases the action of DNA gyrase and topoisomerase IV,

with the resultant effect of low level fluoroquinolone resistance (Strahilevitz et al. 2009, Aldred et al. 2014). Among the most common of these proteins are *qnrB* genes, which are derived from chromosomes of different *Citrobacter* species (Jacoby et al. 2011), *qnrA* genes derived from *Shewanella* algae, *qnrD* genes mostly linked to small plasmids in *Proteus mirabilis*, and *qnrS* genes common in *Vibrio splendidus* (Poirel et al. 2005). Both *qnrA* and *qnrB* occur frequently on class 1 integrons where they are co-carried in association with other resistance determinants (Robicsek et al. 2006a). The -cr (ciprofloxacin resistance) variant of the *aac(6,)-Ib* (*aacA4*) gene encodes an enzyme that can N-acetylate the piperazinyl substituent of ciprofloxacin and is associated with low-level fluoroquinolone resistance but with reduced resistance to aminoglycosides (Robicsek et al. 2006b). Two plasmid-encoded efflux pumps are also associated with fluoroquinolone resistance. The *qepA* gene encodes a proton antiporter efflux pump of the major facilitator superfamily (MFS) and expels quinolones from the cell (Yamane et al. 2007). The *OqxAB* efflux pump encoded by *oqxAB* genes initially identified on a plasmid apparently been captured from the *K. pneumoniae* chromosome contributes to resistance to olaquindox (which is used as a growth promoter in animals) and to fluoroquinolones and chloramphenicol (Hansen et al. 2007). **Table 4** shows the main types of plasmid mediated quinolone resistance determinants in Enterobacterales (Partridge 2015).

Table 4. Main types of Plasmid-mediated quinolone resistance determinants in Enterobacterales

Gene	Variant	Mobile element	Source
qnrA	7	ISCR1	*Shewanella* algae
qnrB2		ISCR1	*Citrobacter* spp.
qnrB1		IS3000, IS26	*Citrobacter* spp.
qnrB4		ISCR1	*Citrobacter* spp.
qnrB6		ISCR1	*Citrobacter* spp.
qnrB10		ISCR1	*Citrobacter* spp.
qnrB19		ISEcp1	*Citrobacter* spp.
qnrD	2	Small plasmid	Unknown
qnrS1-like	6	IS26	*Vibrio splendidus*
qnrS2-like	2		Unknown
qnrS5-like	1		Unknown
qnrVC	6	Gene cassette	Vibrio
aac(6')-Ib-cr	2	Gene cassette	Unknown
qepA	2	ISCR3, IS26	Unknown
oqxAB	1	IS26	*K. pneumoniae*

2.11.3 Sulfonamides and trimethoprim

The combination of sulfamethoxazole and trimethoprim acts by interfering with the two successive steps in folate biosynthesis. Sulfonamide resistance is achieved by genes encoding drug-resistant dihydropteroate synthases, *sul1*, *sul2*, or *sul3*. The *sul1* gene forms part of the 3′-conserved segment of class 1 integrons and is often transmitted together with other ARGs occurring on gene cassettes in the variable region (Recchia and Hall 1995). The *sul2* gene frequently occurs on plasmids that harbor other ARGs. The *sul3* gene is often associated with unusual class 1 integrons and has been linked to the macrolide resistance gene *mef* (B) (Sunde et al. 2008, Liu et al. 2009, Siqueira et al. 2016). Trimethoprim resistance genes are categorized as *dfrA* and *dfrB*, with at least 19 different *dfrA* variants and less than 8 different *dfrB* gene cassettes, which encode trimethoprim-resistant dihydrofolate reductases. These genes were described in Enterobacterales and other Gram-negative bacteria (Partridge et al. 2009a). Most *dfrA* and *dfrB* genes occur on gene cassettes integrated into class 1 or class 2 integrons. A few other *dfrA* genes are associated with ISCR1 or ISCR2 elements. The occurrence of a *dfrA* gene linked with ISCR1 and *sul1* in the 3'-CS in a class 1 integron can confer resistance to trimethoprim/sulfamethoxazole (cotrimoxazole). *Sul3* is linked to a type of class 1 integron only known to be associated with a gene cassette that includes *dfrA12* (Partridge et al. 2009b).

2.12 Plasmid-mediated resistance and multi-resistance

Bacterial plasmids serve a key role as potential vehicles for acquiring resistance genes and subsequent delivery to recipient hosts. Some resistance plasmids are broad host range and can be transferred among various species through bacterial conjugation, whereas narrow host range plasmids are transferred among a small number of cells from similar bacterial species. Plasmid-encoded antibiotic resistance is conferred to different classes of antibiotics that are currently in use as frontline drugs during clinical treatments such as β-lactams, fluoroquinolones, and aminoglycosides (Bennett 2008). Plasmids that harbor resistance genes may either be large conjugative plasmids, which carry the genes encoding all of the machinery required to transfer themselves between bacterial cells or may be smaller mobilizable plasmids that may be co-transferred by conjugative plasmids. These plasmids generally consist of a 'backbone' that encodes plasmid functions, which include replication, stability, and conjugation, into which variable

‘accessory’ regions are inserted. Accessory regions, such as those encoding antibiotic resistance, may occupy a large proportion of the plasmid. When essential plasmid functions are disrupted, a mobile element that has become inserted without incapacitating a plasmid may become a target of insertion of other mobile elements and associated resistance genes, such elements are often found clustered together in complex multi-resistance regions (MRR) (Partridge 2011). Subsequent transfer of resistance genes between DNA molecules are then likely to be facilitated by other nearby mobile elements or by homologous recombination between repeated elements, which may also result in rearrangements in MRR. Clustering of resistance genes in this fashion implies that certain combinations of genes frequently travel together and that use of one antibiotic is likely to select for maintenance of genes giving resistance to unrelated antibiotic classes. For instance, *E. coli* ST131 isolates often carry $bla_{CTX\text{-}M\text{-}15}$ in large, complex MRR on IncF-type plasmids. These related MRR may carry different combinations of the $bla_{TEM\text{-}1}$, *aac(3)-II*, *tetA(A)* (tetracycline resistance), and either both *aac(6’)-Ib-cr* and $bla_{OXA\text{-}30}$ or none of these genes (Partridge et al. 2009a). *P. aeruginosa* resistance via horizontal gene transfer has been described for the genes encoding β-lactam-hydrolyzing enzymes (extended-spectrum β-lactamases and the carbapenemases), aminoglycoside-modifying enzymes, 16S rRNA methylases that result in high-level pan-aminoglycoside resistance (Poole 2011). It is of great concern that transferable plasmids harboring some of the resistance genes are mobile among a wide range of unrelated Gram-negative bacteria which increases the antimicrobial resistance transfer rate (Hong et al. 2015). Acquired antibiotic resistance genes in bacteria are frequently carried on plasmids with F plasmids being the most common conjugal plasmids linked to antibiotic resistance ("R factors") (Moran et al. 2015). According to Stephens et al. the majority of antibiotic resistance genes are associated with F replicons, and in most cases, multiple subtypes of F replicons occur on the same plasmids. Antibiotic resistance genes with a narrow host range have been identified including IncI complex replicons (Z, B/O, K, or I1). F- and I-complex replicons are typically associated with conjugating plasmids (Stephens et al. 2020). ESBL genes, carbapenemase genes, genes coding aminoglycoside-modifying enzymes and plasmid-mediated quinolone resistance (PMQR) genes are the most frequently described resistance genes on IncF plasmids (Rozwandowicz et al. 2018).

2.13 Tools to assess antibiotic resistance in wastewater and other aqueous environments

Identifying hotspots of possible ARGs dissemination starts by determining the relative occurrence of antibiotic resistant bacteria or ARGs in the wastewater network. Two main approaches are followed, either culture-based or molecular based. The molecular based approaches have been extended to the detection of genetic structures involved in ARG capture namely integrons, enhancing the understanding of ARG dynamics in complex anthropogenic environments.

2.13.1 Characterization by cultivation-based methods

Antibiotic resistance testing is highly standardized worldwide, enabling laboratories to assist clinicians in bacteria infection therapy (Wayne 2010). Given the nature of the samples involved, numerous modifications have been introduced in order to achieve reliable and accurate methods with a feasible application to surface or wastewaters (Novo and Manaia 2010). Although culture-independent approaches have been used, the determination of prevalence values and resistance patterns is more frequently based on culture-dependent methods (Czekalski et al. 2012) These methods although with several adaptations, are supported by guidelines developed for clinical and veterinary microbiology (Andrews and Testing 2009). The use of selective culture media allows the enumeration of viable cells and isolation of specific bacterial groups. After purification the isolates can be identified and typed for their antibiotic resistance patterns allowing the calculation of resistance rates or the definition of resistance profiles and multidrug resistance phenotypes (Garcha et al. 2016). Antibiotic resistance testing is frequently based on the disc diffusion or microdilution methods which according to standardized values allow the distinction between resistant and susceptible organisms (Rizzo et al. 2013a). Culture-dependent methods can survey microorganisms that are viable at a given moment and for which the growth conditions are known. In spite of skipping non-culturable microorganisms culture dependent methods have the advantage of permitting the phenotypic characterization of isolates relevant to assess for instance their profile of multidrug resistance, minimum inhibitory concentration of different antibiotics, antibiotic resistance spectrum or the capability to take part in horizontal gene transfer processes. However, depending on the environment, less than 1-10% of bacteria can be culturable with the largest

majority of environmental bacteria falling permanently or transiently within the wide category of non-cultural microorganisms (Ivone et al. 2013).

The cell cultivation allows the possibility of determining phenotypic traits, many of which are crucial for understanding the ecology of a given bacterial group. Incase of antibiotic resistance, this knowledge is the basis to assess the propagation or gene transfer potential of specific ARB under environmental conditions. In this aspect, relevant phenotypic traits that can be examined in bacterial isolates include metabolism (e.g. carbon source utilization, identification of auxotrophies), required physico-chemical conditions for growth (e.g. PH, temperature or salinity), biofilm formation capacity, sporulation, motility, tolerance against stressful conditions among others (McLain et al. 2016). In addition, the analysis of ARB isolates facilitates the analysis of the harbored ARGs as well as the mobile genetic elements to which they are associated and above all supports the identification of the species or clones that may be of major relevance for the spread of a given ARG (Kaplan et al. 2015, Hembach et al. 2017). Culture based methods are highly laborious and time consuming and alternative adapted techniques have been developed (Ferreira da Silva et al. 2007). One of such adaptations involves the use of selective culture media supplemented with antibiotics at concentrations similar to or above those reported as inhibitory for the target bacteria. In this case, the percentage of resistance can be estimated as the ratio between the number of bacteria growing in the presence and in the absence of antibiotic (Novo and Manaia 2010). This method also promotes the enrichment of ARB, and therefore can help on the detection of resistance genetic determinants.

2.13. 2 Molecular biology techniques to assess the diversity of ARB/ARGs in wastewater

Molecular biology methods have been used for several years for the examination of water of different origins and qualities (Alexandrino et al. 2007). These techniques may be applied to identify specific DNA targets without prior cultivation of the organisms. They can be used to detect microorganisms that cannot be grown in the laboratory or that multiply very slowly but contribute to the resistance in specific compartments (Trevors 2011). Genetic tests aim at detecting resistance genes or genetic elements contributing to horizontal gene transfer in bacterial isolates and communities by using DNA probes or PCR methods. Genetics based tests are more accurate than antibiograms in tracing the epidemiological spread of relevant resistance genes in a hospital or

wastewater community setting since beside phylogenetic DNA markers, other genetic markers like antibiotic resistance and virulence genes could be detected in parallel. Natural habitats harbor many non-culturable bacteria or bacteria of different physiological states which limit cultivation-based quantitative analyses.

The culture-independent approaches rely primarily on the extraction of genetic material most of the times DNA and not so often RNA from a sample. The DNA or RNA extraction efficiency may vary with the protocol used as well as the matrix to analyse. The protocol may favor the extraction of nucleic acid from specific bacterial groups (e.g Gram negative in comparison to Gram positive) or it may be more or less effective on the removal of inhibitors (McCarthy et al. 2015, Li et al. 2018). While DNA based analyses are the best choice for gene survey, RNA-based analyses are the option when gene expression rather than gene presence is to be investigated. Molecular based methods offer the great advantage of getting rid of biases associated to the non-cultivability of some ARB or the non-expression of ARG in some bacterial hosts (Trevors 2011).

2.13.3 Quantitative PCR approach

Two major approaches, the quantitative PCR (qPCR) and the metagenomics have been used to assess the wastewater resistome. Due to its potential for quantitative and highly specific analyses, the qPCR has been increasingly used, with an ever-increasing number of recommended primers and reference conditions (Czekalski et al. 2015, Rocha et al. 2020). The use of qPCR array is in this respect an important advance allowing the simultaneous analyses of a large number of genes (Karkman et al. 2016). qPCR is limited by impossibility to design primers for new or unknown genes, since the primers design is based on reference sequences that are already described and deposited in the databases. Since primers only require homology with a small fragment of DNA, annealing may occur with non-target genes with regions of high identity with the primers. However, these false positive reactions can be easily identified based on melting temperature of the amplicon (Rocha et al. 2020). Other challenges of qPCR analyses of environmental samples refer to different types of often unknown or unexpected interferences or inhibitor agents (e.g humic acids), which can reduce the accuracy of the process (Sidstedt et al. 2015). Quantitative PCR assays tracking various resistance genes originating from different pathogens have revealed their spread

in clinical and municipal wastewater systems and their occurrence in surface water and drinking water biocoenosis (Lupo et al. 2012) Many studies have demonstrated the presence of clinically relevant antibiotic resistance genes along the water cycle, with potential hotspots of wastewater discharge into other aquatic environments (Zhang et al. 2009a). As an example, methicillin resistance gene *mecA* of Staphylococci, the β-lactam resistance gene *ampC* of Enterobacterales, the carbapenem resistance gene bla_{VIM} of *Pseudomonas aeruginosa* and the vancomycin resistance gene *VanA* of Enterococci as well as taxon specific ribosomal DNA sequences for Enterococci and *P. aeruginosa* have been chosen as targets to quantify their abundances in defined amounts of DNA extracts of different wastewater matrices (Schwartz 2012).

2.13.4 Metagenomics approach

Metagenomics approach is becoming a widely applied method for some routine analyses of ARGs in various environments. Additionally, similarity searching-based annotation may give a more comprehensive profile of ARGs than the qPCR method which is limited by the availability of the primers designed so far. This approach has the potential to provide not only an overview of the already known ARGs but also of their variants or possible new ARGs that may exist in a given environment (Oulas et al. 2015). In metagenomics analyses, the availability of representative databases from which it is possible to extract reliable information may also constitute a bottleneck due to the limited size, phylogenetic and geographic coverage of the databases (Bengtsson-Palme et al. 2017, Arango-Argoty et al. 2018). The usefulness of metagenomics to study ARGs and bacterial diversity in several environments including wastewater has been widely demonstrated (Li et al. 2015, Munck et al. 2015). The approach may have a limited capacity to explore, if the abundance of a given ARG decreases with the treatment, an extremely rare ARG exists in a given sample or different ARGs variants (polymorphisms) with distinct clinical relevance are in the same sample. In addition, the lack of standard methods or universal tools to be used in metagenomics analyses may represent a constraint for the analyses of reproducibility and comparability of data obtained in different occasions or places (Escobar-Zepeda et al. 2015). Since DNA is extracted from the whole community followed by qPCR, this approach is useful for the detection and quantification of antibiotic resistance and pathogen targets even of non-culturable fractions of populations (Khan and Yadav 2004). In case of underrepresented ARG in the metagenome of

complex microbial communities the process of nucleic acid extraction and its quality are very crucial for the subsequent PCR gene detection. Wastewater samples with a variety of undefined natural and anthropogenic substances appear to be difficult matrices for a microbiological analysis at the nucleic acid level. Despite the specific extraction and purification techniques, inhibitors like humic substances, organic salts or detergents disturb the detection of specific bacterial species and functional genes (Volkmann et al. 2007). Apart from the exigency of an effective extraction method that reduces interfering impurities, further interactions of the sample matrix DNA with the PCR have to be considered when qPCR is applied to wastewater samples.

3.0 AIMS

The main aim was to isolate and characterize the extended-spectrum β-lactamase (ESBL) and carbapenemase-producing Gram-negative opportunistic pathogens belonging to the order Enterobacterales and *Pseudomonas aeruginosa* from hospital effluents, urban wastewater and wastewater treatment plant (influent, activated sludge reactor and digested sludge) as well as to determine the presence and concentration of selected antibiotic compounds in the same wastewater samples.

In order to achieve this aim, the study was guided by the following objectives.

1. Determine the presence and concentration of various antibiotic compounds in hospital effluents, wastewater treatment plant and urban wastewater.
2. Isolate ESBL and carbapenenamase-producing members of Enterobacterales (*Escherichia coli*, *Klebsiella* spp., *Enterobacter,* and *Citrobacter* species) and *Pseudomonas aeruginosa* from the wastewater.
3. Investigate the antimicrobial resistance profiles and determine the presence of ESBLs and metallo-β-lactamases (MBLs) among the broad-spectrum β-lactam resistant isolates phenotypically.
4. Molecular genotyping of common ESBLs (CTX-M, TEM, and SHV*)* and carbapenamases (IMP, VIM, NDM, SPM, KPC, OXA-48) among the broad-spectrum β-lactams ($3^{rd.}$ generation cephalosporins and carbapenems) resistant isolates of Enterobacterales and *P. aeruginosa.*
5. Comparative sequence analysis of the plasmid DNA of selected multiple antibiotic-resistant isolates of *Escherichia coli, Klebsiella* spp. and *C. freundii* to identify the gene variants of the ESBL and other plasmid encoded resistance mechanisms.
6. Sequence and identify the bla_{VIM} gene variant prevalent in carbapenem-resistant isolates of *P. aeruginosa* and possible mutations among the VIM sequences of selected isolates.

4.0 MATERIALS AND METHODS

4.1 Study sites and sampling strategy

This study was carried out in the city of Pécs, in southwest Hungary. Wastewater samples were drawn from four hospital wastewater discharge points, H1 (387 beds), H2 (106 beds), H3 (127 beds), and H4 (348 beds), a discharge point of a nursing home for the elderly (NH, 490 beds), urban wastewater sewer lines (UWW1-2), and a WWTP **(Figure 9**). Effluent samples from the healthcare facilities were collected directly from two separate generation points serving different buildings before joining the main sewer pipe. Samples from the WWTP were collected from the influent directly behind the grating screen, the activated sludge reactor, and the digested sludge after mesophilic digestion. The urban wastewater was collected 4 km upstream of the health care facilities (UWW1) and at a second spot upstream of the WWTP (UWW2). A 30 ml sample was collected every 15 minutes by lowering a flask into the wastewater flow over a period of 4 h and the aliquots pooled to constitute 480 ml composite sample in sterile 500 ml glass bottles. One grab sample was drawn from the activated sludge reactor and the digested sludge. Samples were transported on ice to the laboratory and stored at 4^0C before assaying within 6 hours. The WWTP processes wastewater from the central business district, health care facilities, domestic wastewater, and some storm runoff and serves a population equivalent to slightly over 200000 inhabitants. The wastewater treatment involves three stages (primary clarification, secondary-activated sludge system, and mesophilic sludge digestion, tertiary-UV treatment) with the final effluent discharged into the nearby surface stream. The study was conducted from March 2019 to March 2020 with each sampling spot visited thrice.

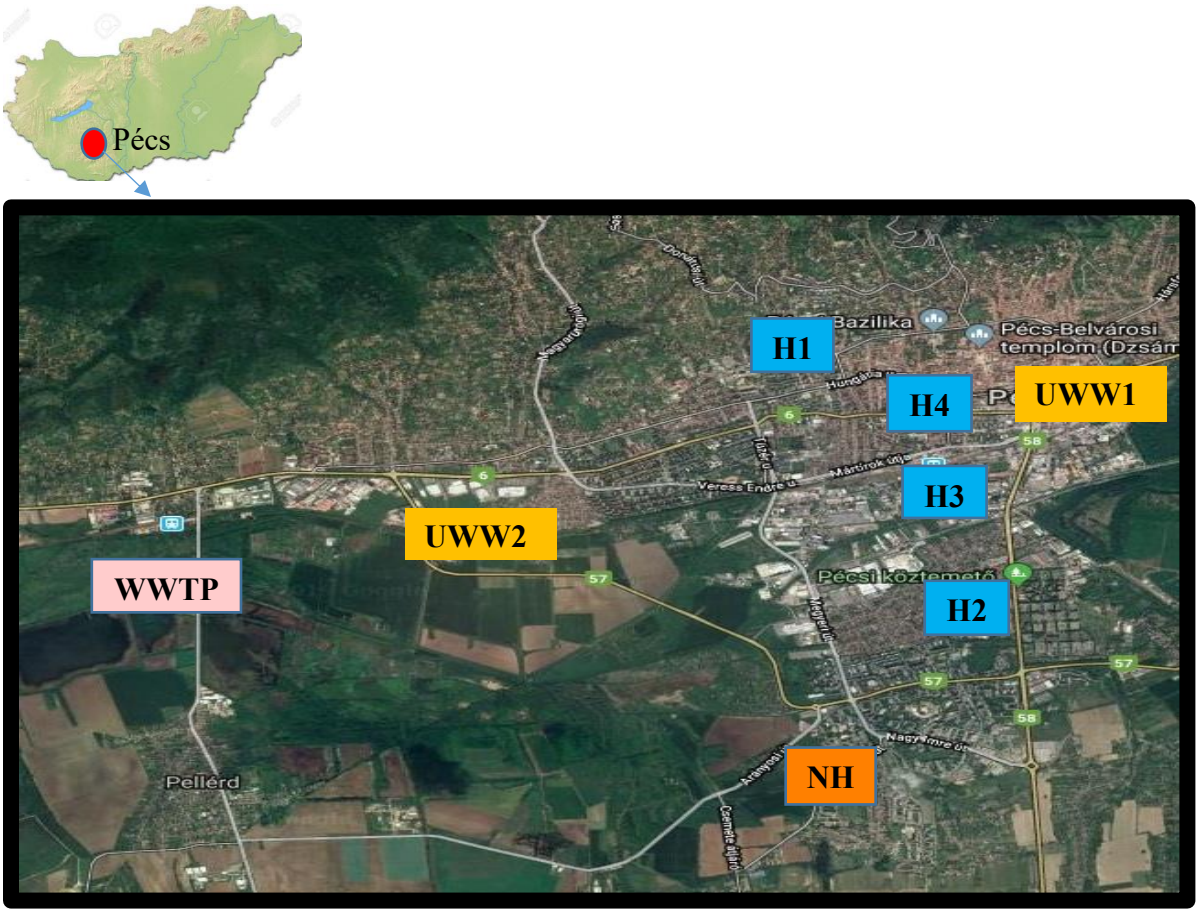


Figure 9. A section of the map of the city of Pécs showing the sampling locations. **Study sites:** H1-H4: hospital effluent, NH: nursing home, UWW: urban wastewater, WWTP: wastewater treatment plant.

4.2 Sample preparation and LC-MS analysis of antibiotic molecules in wastewater

Clarification of the raw wastewater samples was carried out by centrifugation for 10 min at 10000 × *g* at 4°C using Ohaus FC5718R centrifuge (Ohaus Europe Gmbh). After centrifugation, supernatants were collected and acidified to pH, 3.0 with formic acid (98% m/m, Lach Ner). Samples were vacuum filtered, initially through a GF/A 1.6 µm glass microfibre filter (#1820-047, Whatman), and subsequently, through a GF/F 0.7 µm glass microfibre filter (#516-0345, VWR). Sample extraction was performed by Oasis PRiME HLB (200 mg, 6 ml; Water) cartridges using an automated SPE system (Dionex AutoTrace 280, Thermo Scientific). After loading 100 ml of samples, cartridges were washed with 5% methanol in 3 ml water. The cartridges were dried with nitrogen gas to eliminate the aqueous residues. Elution was performed by 5 ml of 100% methanol

(UPLC grade; Sigma Aldrich). Eluates were evaporated to dryness by nitrogen gas stream and reconstituted with 1000 ml of acetonitrile:water 10:90 (v/v). Instrumental analysis was carried out by a Waters ACQUITY UPLC H-Class System, coupled with a Xevo TQ-S micro triple quadrupole mass spectrometer equipped with an electrospray ionization source operated in positive ion mode. The chromatographic separation was performed on the Waters ACQUITY UPLC BEH C18 column (50×2.1mm i.d., 1.7 µm particle size). The injection volume was 10 µl. The mobile phase consisted of ultrapure water (A) and UHPLC-MS grade methanol (B) both containing 0.1% LC-MS grade formic acid at a flow rate of 0.4 ml/min. The gradient program was started with 10% B from 0-2 min, followed by a linear increase to 90% B in 8 min and kept for 2 min, then back to initial condition and held for 3 min to allow re-equilibration. The total run time was 15 min.

4.3 Enumeration of heterotrophic aerobic plate count bacteria, total and antibiotic resistant Gram-negative bacteria

Determination of the total number of live, aerobic bacteria in the wastewater samples was done on plate count agar (PCA). Eosin Methylene Blue (EMB) Agar (Biolabs, Budapest, Hungary) containing ceftriaxone (CRO, 2.0 µg $ml^{-1;}$ Merck, Darmstadt, Germany), or imipenem (IMP, 8.0 µg ml^{-1} ; Merck, Darmstadt, Germany) was used to enumerate the antibiotic resistant Gram-negative bacteria and to select for Enterobacterales. The antibiotics were dissolved in their recommended diluents and filter sterilized through a 0.45 µm cellulose-acetate filter before addition to the media. EMB agar plates devoid of antimicrobials served as controls for determining the total Gram-negative bacteria count respectively. A 5 ml subsample drawn from homogenized 480 ml sample was serially diluted in phosphate buffered saline (PBS) containing 0.1% tween 80 up to 10^{-3} dilution and volumes of 50 µL was drawn from each dilution and plated on freshly prepared PCA and EMB media in triplicate. The plates were incubated under aerobic conditions at 35 ± 2 ^{0}C and evaluated for colony formation after 24 and 48 h (Aali et al. 2014). Dilutions with 20-200 colony forming units (cfu) were enumerated and the number of bacteria expressed as colony forming units per mL (cfu ml^{-1}) while the resistance rate for each antibiotic corresponded to the ratio of cfu ml^{-1} on the culture medium with and without antibiotic (Novo and Manaia 2010, Berendonk et al. 2015).

4.4 Isolation and identification of enteric bacteria and *P. aeruginosa*

In each sampling series, up to 5-10 colonies, representatives of different colony morphotypes (colony contour, color, pigment production, or size) were randomly picked from each culture medium. Lactose fermenting colonies of presumptive enteric bacteria included typical green metallic sheen colonies (*E. coli*), large mucoid pinkish colonies (*Klebsiella* spp), and pink to purple colonies (*Enterobacter* spp) (Cheesbrough 1990). Cetrimid Nalidixic (CNA) Agar (Biolabs, Budapest, Hungary) supplemented with 10 ml glycerol and containing ceftazidime (CAZ, 8.0 µg ml^{-1}; Merck, Darmstadt, Germany) was used to select for *P. aeruginosa.* Blue green and yellow green colonies characteristic of pyoverdine and pyocyanin pigments were representative of *P. aeruginosa*. All the isolates were sub-cultured on nutrient agar, incubated at 35 ± 2 ^{0}C for further 18-24 hours and identified with for MALDI-TOF MS.

4.5 MALDI sample preparation by direct analysis method and MALDI-TOF MS measurement

One colony of each bacterial isolate, subcultured for 24 h, was transferred onto a target plate with a sterilized toothpick and left to air dry at ambient temperature. 1 µL MALDI matrix solution of alpha-cyano-4-hydroxycinnamic acid (HCCA) (Sigma-Aldrich) was overlaid on each sample well and allowed to air dry for several minutes before analysis. The MALDI target plate was placed into the MALDI-TOF mass spectrometry for automated measurement and data interpretation. All samples were measured in duplicate using a Microflex LT MALDI-TOF mass spectrometry (Bruker Daltonics, Bremen, Germany) in positive linear mode across the m/z range of 2 to 20 kDa; for each spectrum, 240 laser shots at 60 Hz in groups of 40 shots for each sampling area were collected. The MALDI Biotyper RTC 3.1 software and the MALDI Biotyper Library 3.1 were used for spectrum analysis. Score values of $\geq$ 2.0 were considered reliable identifications (Blondiaux et al. 2010). The cultures were preserved at −80 ^{0}C in nutrient broth supplemented with 20% glycerol.

4.6 Antimicrobial susceptibility profiles and phenotypic detection of β-lactamases

Antimicrobial susceptibility was established using the standardized disk diffusion method on Mueller Hinton agar (Biolabs, Budapest, Hungary) according to EUCAST 2018 guidelines. The standard antibiotic discs belonging to the following classes were used: (1) β-lactams; ceftriaxone (CRO, 30 µg), ceftazidime (CAZ, 10 µg), cefotaxime (CTX, 30 µg), cefpodoxime (CPD, 10 µg), cefoxitin (FOX, 30 µg), imipenem (IMP, 10 µg) and meropenem (MEM, 10 µg), (2) aminoglycoside; gentamicin (GN, 10 µg), (3) fluoroquinolone; ciprofloxacin (CIP, 5 µg) and (4) sulfonamide; sulfamethoxazole/trimethoprim (SXT, 1.25/23.75 µg) (Oxoid, Wesel, Germany). Quality control was carried out using *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 180112, *Enterobacter cloacae* ATCC 180083, and *P. aeruginosa* ATCC 27853 as wild type negative controls while *E. coli* ATCC 151006, *Klebsiella pneumoniae* ATCC 180111, *Enterobacter cloacae* ATCC 161002, and *P. aeruginosa* ATCC 153006 were control strains with known resistance phenotype. Inoculum's concentration was standardized to 0.5 McFarland turbidity and plates incubated for 18-20 h at 35 ^{0}C were evaluated for the formation of zones of inhibition. The zone diameters were interpreted according to the European Committee on Antimicrobial Susceptibility Testing clinical breakpoints, version 8.1, 2018 (EUCAST 2018). Multidrug resistance among the strains was defined as resistance to three or more antibiotic classes. A combined disk test was used to screen for the production of extended-spectrum β-lactamase and metallo-β-lactamase. Cefotaxime (CTX 30 µg), cefpodoxime (CPD 10 µg), imipenem (IMP 10 µg), and meropenem (MEM 10 µg) disks were placed next to the disks with cefotaxime/clavulanic acid (CTC, 30/10 µg, CTC, 40), cefpodoxime/clavulanic acid (10/10 µg, CD, 01), imipenem/ ethylenediamine tetraacetic acid (EDTA) , (IMP 10 µg / EDTA 292 µg - IEL 292), and meropenem/ EDTA (MEM 10 µg /EDTA 292 µg - MEL 292), respectively (Oxoid, Wesel, Germany) on Mueller Hinton agar plates (Biolabs, Budapest, Hungary) with an inoculum of 0.5 McFarland. An increase in the inhibition zone size to ≥ 5 mm with the combined disks compared to the disk of cephalosporin/carbapenem alone was considered a positive test for β-lactamase production (Oduro-Mensah et al. 2016).

4.7 Molecular typing of extended spectrum β-lactamases and carbapenemases

Plasmid DNA was isolated using Monarch plasmid DNA miniprep kit according to the manufacturer's instructions (New England Biolabs T1010, Ipswich, Massachusetts, USA). DNA was isolated from freshly grown pure colonies transferred into Luria Bertani broth and incubated in an orbital shaker at 35 ^{0}C and 200 rpm for 12–16 h. 1.5 ml bacterial culture was transferred to 2 ml microfuge tubes and pelleted by centrifugation at 16,000 × g for 30 seconds and the supernatant was discarded. The pellet was resuspended in 200 µL pH Tris plasmid resuspension buffer (B1) containing EDTA and vortexed for complete resuspension. The debris was lysed by adding 200 µL plasmid lysis buffer (B2) containing sodium hydroxide and sodium dodecyl sulfate and the tubes inverted gently 5-6 times until the color changed to dark pink and the solution became clear and viscous before incubating for 1 minute on ice. The lysate was neutralized by adding 400 µL of plasmid neutralization buffer (B3) containing 3.0 M potassium acetate, pH 5.0. The tubes were gently inverted to form a uniformly yellow precipitate and incubated for 2 minutes and the lysate was clarified by spinning for 5 minutes at 16,000 × g. The supernatant was carefully transferred to the spin column and centrifuged for 1 minute with the flow through discarded. The spin column was re-inserted into the collection tube and 200 µL of Plasmid wash buffer 1 was added to remove RNA, proteins, and endotoxin and centrifuged for 1 minute. 400 µL of Plasmid Wash Buffer 2 containing ethanol was added for a second wash and centrifuged for 1 minute to remove trace components, including salts. The spin column was then transferred to a clean 1.5 ml microfuge tube. 30 µL DNA elution buffer containing sodium chloride, Tris-CL and isopropanol was preheated to 50 ^{0}C to improve the DNA yield and added to the center of the column to ensure the matrix is completely covered for maximal efficiency of elution and after a 1 minute incubation at room temperature, the DNA was eluted by spinning for 1 minute. DNA yields and purity were measured using a Nanodrop spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, USA) and stored at –20 ^{0}C for subsequent PCR amplification.

4.8 PCR amplification of template DNA

PCR reactions for target genes belonging to *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-48}$, *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{NDM}$, and *bla*$_{SPM}$ were performed in a final volume of 25 µL containing 1 µL template

DNA, 1.0 µM, Forward primer, 1.0 µM, Reverse primer, 12.5 µL DreamTaq PCR master mix (2x) containing Dream Taq DNA polymerase, optimized 2x Dream Taq buffer, 4.0 mM, $MgCL_2$, 0.4 mM each of dATP, dCTP, dGTP and dTTP, (Thermo Scientific, Waltham, Massachusetts, USA) and by addition of nuclease free water. Conventional PCR assay was used and the amplification thermal profile was applied as follows: Initial denaturation at 95 ^{0}C for 2 minutes, repeated 35 times with a cycle of 95 ^{0}C for 30 seconds, 30 seconds at the appropriate primer annealing temperature for the specific primer, primer extension at 72 ^{0}C for 1 minute, and final elongation at 72 ^{0}C for 10 minutes, with a holding step at 4 ^{0}C. Gly238→Ser mutation associated with the hydrolysis of third-generation cephalosporins was identified through digestion of *bla*$_{SHV}$ PCR product with *Nhe*I (New England Biolabs). Colony PCR was performed using OneTaq quick load Mastermix to confirm the presence of chromosomally encoded β-lactamases among carbapenem resistant isolates whose plasmids were not obtained. Discrete colonies were dipped into the reaction tubes containing 25 µL One Taq master mix (New England Biolabs, Budapest, Hungary) PCR primers and nuclease free water. Thermal cycling conditions were initial denaturation at 94 ^{0}C for 2 min, 35 times repeated cycle of 94 ^{0}C for 30 s, 30 s at the appropriate primer annealing temperature for the specific primer, primer extension at 68 ^{0}C for 1 min, and final elongation at 68 ^{0}C for 10 min, with a holding step at 4 ^{0}C. Post PCR analysis was performed in 1.5% (w/v) agarose gel stained with 2 µL serva DNA stain G (Bio-Connect, Begonialaan, Netherlands). The DNA amplification was done using the primer sequences shown in **Table S3**.5 µL of each DNA sample was mixed with 2 µL of 6× loading dye and electrophoresed in 1× Tris - EDTA (TAE) running buffer at 100 V for 1 h and visualized under ultraviolet transilluminator. 10 µL of either phage Lambda DNA digested with *Eco*RI/*Hind*III (21226 bp) and a low range molecular weight marker (700 bp) (Thermo Scientific, Waltham, Massachusetts, USA), or both in some cases were included in each run as DNA size markers. In each PCR run, a positive control consisting of a clinical isolate of a confirmed reference strain was included for each genotype. The control strains were well characterized clinical isolates kindly provided by the microbiology laboratory of the University of Pécs Medical School (Melegh et al. 2014a). For the colony PCR, 10 µL of each reaction was directly loaded onto an agarose gel alongside a PCR product from an appropriate reference strain and a DNA ladder.

4.9 Plasmid DNA library preparation, sequencing and analysis

Selected isolates (*E. coli* n = 10, *K. pneumoniae* n = 9, *K. oxytoca* n = 1, and *C. freundii* n = 1) were subjected to NGS sequencing. The selection was based on antimicrobial susceptibility profiles and site of isolation. The library for NGS sequencing was prepared using Swift 2S Turbo DNA Library Kits (Swift Biosciences, Ann Arbor, Michigan, USA). Briefly, 100 ng genomic DNA was fragmented, end prepped, and adapter ligated. Magnetic bead size selection was performed to select 250–300 bp insert size fragments, followed by the library amplification according to the manufacturer's instructions. The quality of the library was checked on the 4200 TapeSation System using D1000 Screen Tape (Agilent Technologies, Palo Alto, CA, USA) and the quantity was measured on Qubit 3.0 (Thermo Scientific, Waltham, MA, USA). Illumina sequencing was performed on the NovaSeq 6000 instrument (Illumina, San Diego, CA, USA) with a 2x151 run configuration. Quality control (QC), trimming, and filtering of 150 bp paired-end raw reads were performed in the preprocessing step. The QC analysis was performed with FastQC (Andrews et al. 2020). The Phred-like quality scores (Qscores) were set to >30. Poor quality reads, adapters at the ends of reads, limited skewing at the ends of reads were eliminated by using Timmomatic (Bolger et al. 2014). Since data contained genomic DNA debris, identification of plasmid derived contigs was performed after de novo assembly of cleaned reads. For plasmid identification, genes characteristically encoded in plasmids for each strain were determined based on literature and by aligning them for the contigs using locally the Blast+ (Altschul et al. 1990). Prokaryotic gene finding was performed by Glimmer using the Bacterial, Archaeal, and Plant Plastid Code. Glimmer uses Interpolated Markov Models (IMMs) to identify the coding regions and to distinguish them from non-coding DNA, which enabled identified genes to be annotated (Delcher et al. 1999). Functional annotation and Gene Ontology (GO) analysis were carried out using OmixBox.Biobam as follows: sequences were blasted against the NCBI nr (non-redundant) database (taxID: 2Bacteria), applying blastn configuration locally. To retrieve GO terms associated with the 10 Hits obtained by the Blast search GO mapping and annotation were performed. GeneBank identifiers (gi), the primary blast Hit ids, were used to retrieve UniProt IDs making use of a mapping file from PIR (Non-redundant Reference Protein Database), including PSD, UniProt, Swiss-Prot, TrEMBL, RefSeq, GenPept, and PDB. Accessions were searched directly in the dbxref table of the GO database. BLAST result accessions were searched directly in the gene-product

table of the GO database; GO annotations were specified according to GO terms: molecular function, cellular component and biological process (Götz et al. 2008). For detection of antimicrobial resistance genes and identification of plasmid incompatibility groups ResFinder 4.1 and PlasmidFinder 2.1 were used (Carattoli et al. 2014, Bortolaia et al. 2020). Each contig of all isolates was aligned with MUMmer 4.0 in order to identify similar regions (Marçais et al. 2018). MUM indices were calculated pairwise, and the resulting distance matrix was used to cluster the isolates with neighbor joining method (Deloger et al. 2009). Visualization of clusters was performed with Treesplits (Huson and Bryant 2006).

4.10 Isolation of genomic DNA from *P. aeruginosa* for amplification of VIM gene

Genomic DNA was extracted using Macherey-Nagel NucleoSpin Microbial DNA kit according to the manufacturer's instructions (Waltham, Massachusetts, USA). Pure colonies of *P. aeruginosa* on Mueller Hinton agar were transferred into 20 ml Luria Bertani broth in 100 ml flasks and incubated in an orbital shaker at 37 ^{0}C and 200 rpm for 12-16 h. 1 ml bacterial culture was transferred into 2 ml microfuge tubes, the cells were harvested by centrifugation at 8,000 × g for 30 seconds and the supernatant discarded. The cells were resuspended in 100 µL elution buffer (BE). The cell suspension was transferred into MN bead tubes where 40 µL buffer (MG) and 10 µL liquid proteinase K were added. The MN tubes were agitated for 4 min in a cell disruptor/tissuelyser (Qiagen, Germantown, Maryland, USA). The MN bead tubes were centrifuged for 30 s at 11,000 × g and mixed with 600 µL buffer (MG) to adjust DNA binding conditions. The MN bead tubes were centrifuged for 30 s at 11,000 x g and 600 µL of the supernatant was transferred into the NucleoSpin Microbial DNA columns placed in 2 ml collection tubes. The samples were centrifuged for 30 s at 11,000 × g and the flow through was discarded with the collection tube. The columns was transferred into fresh 2 ml collection tube and 500 µL buffer (BW) was added to wash the silica membrane. The columns were centrifuged for 30 s at 11,000 x g, the flow through was discarded and the columns were placed back into the collection tubes. 500 µL buffer (B5) was added to the column and centrifuged for 30 s at 11,000 × g, the flow through was discarded and the columns were placed back into the collection tubes. The silica membrane was dried by centrifugation at 11,000 × g for 30 s. The NucleoSpin Microbial DNA column was placed in 1.5 ml nuclease free tube and 100 µL elution buffer (BE) was added. The

column was incubated at room temperature for 1 min and centrifuged for 30 s at 11,000 × g. The DNA was stored at –20 ^{0}C for subsequent PCR amplification of the VIM gene.

4.11 Sequencing of VIM gene

For sequencing of the region of interest, the DNA was amplified using oligonucleotide primers shown in **Table S5**. The primer pairs were designed using the Primer3Plus. The amplified 161 and 775 bp long PCR products were purified using ExoSAP-IT^{TM} PCR Product Cleanup Reagent (Thermo Fisher Scientific Inc., UK) and sequenced in both directions with an Applied Biosystems^{TM} 3500 Genetic Analyzer using BigDye^{TM} Terminator v1.1 Cycle Sequencing Reaction Kit (Thermo Fisher Scientific Inc., UK) according to the manufacturer's instructions. The resultant sequences were compared with the reference sequence of *Pseudomonas aeruginosa* class 1 metallo-β-lactamase (*bla*$_{VIM-4}$) (NCBI GenBank: AY135661.1 and NG_050367.1) using XY genetic program (DNASTAR Inc., Madison, WI, USA). The *bla*$_{VIM}$ gene fragments were amplified from 33 selected samples.

4.12 Statistical analysis

A descriptive statistical analysis (mean, range, and percentage) was performed using Microsoft Excel 2013 (Redmond, WA, USA, Microsoft Corp.). OriginPro version 2016 (Northampton, Massachusetts, USA, OriginLab Corp.) was used for plotting and analysis. Shapiro–Wilk tests were performed to check the normality of variables, while one-way analysis of variance (ANOVA) was used to compare resistance rates among sampling locations. Pairwise t-test was performed to determine differences in resistance rates between hospitals and WWTP. A correlation matrix was used to examine the relationship between β and non-β-lactam antibiotic resistance. P values ≤ 0.05 were considered statistically significant.

5.0 RESULTS

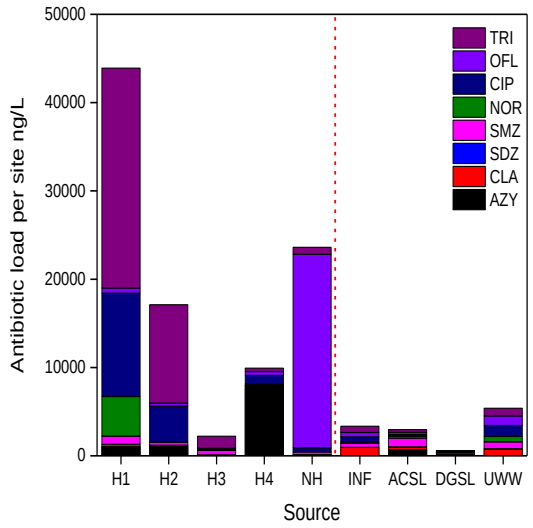
5.1 Prevalence of antimicrobial pharmaceuticals in wastewater

Eight antibiotics belonging to four antimicrobial classes of pharmaceuticals namely macrolides (azithromycin, clarithromycin), fluoroquinolones (ciprofloxacin, norfloxacin, and ofloxacin), sulfonamides (sulphadiazine, sulfamethoxazole) and trimethoprim were recovered at concentrations that varied significantly ($P < 0.05$) among the wastewater samples. Among the antibiotics detected in the highest concentrations were trimethoprim (ranging from 303 ngL^{-1}, activated sludge - 24907 ngL^{-1}, H1), ofloxacin (93 ngL^{-1} digested sludge – 21916 ngL^{-1} nursing home) and ciprofloxacin (131 ngL^{-1}, digested sludge- 11710 ngL^{-1}, H1) (**Table 5**). Sulphadiazine was the lowest detectable antibiotic with a concentration ranging from 5.5 ngL^{-1} H4, - 38.5 ngL^{-1}, urban wastewater). Erythromycin was not detected in any of the samples while tetracycline was either not detected or was below the limit of quantification. A significantly higher concentration of azithromycin ($P < 0.05$) was found in H4 (mean concentration 7957 ngL^{-1}), while clarithromycin was more concentrated in the influent (950 ngL^{-1}) and urban wastewater (768 ngL^{-1}) and ofloxacin was most concentrated in the nursing home (mean concentration 21916 ngL^{-1}). A comparison of the antibiotics concentration between the hospital samples and both the wastewater treatment plant and the urban wastewater samples established significantly higher concentrations of sulphadiazine ($P = 0.008$) in urban wastewater and the wastewater treatment plant, while that of ciprofloxacin and trimethoprim was significantly higher in hospital samples ($P = 0.025$, $P = 0.012$ respectively) (**Figures 10**a - f).

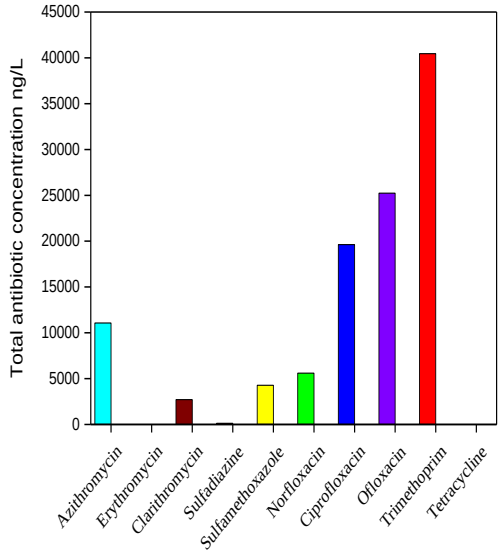
Table 5. Concentrations of target antibiotic compounds in wastewater samples (ngL^{-1}), n=3

Compound	AZY	SDZ	SMZ	TRI	NOR	CIP	OFL	ERY	CLA	TET
Recovery (%)	85	23	29	52	4	17	89	19	41	22
H1	1027	n.d.	943	24907	4475	11710	579	n.d.	267	<LOQ
H2	1149	19.8	342	11148	n.d.	4098	330	n.d.	25.8	<LOQ
H3	5.0	n.d.	511	1353	n.d.	198	43.5	n.d.	111	<LOQ
H4	7957	5.5	21.0	377	141	950	445	n.d.	27.4	n.d.
NH	42.5	18.4	292	794	n.d.	474	21916	n.d.	81.8	n.d.
INF	5.0	13.6	425	705	154	601	495	n.d.	950	<LOQ
ACSL	703	19.9	975	303	210	219	241	n.d.	297	<LOQ
DGSL	178	12.3	n.d.	n.d.	n.d.	131	93.0	n.d.	185	n.d.
UWW	4.9	38.5	767	865	614	1238	1094	n.d.	768	n.d.
F value	354.15	179.90	155.12	338.57	370.32	324.28	380.64		232.78	
P value	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*		0.0001*	

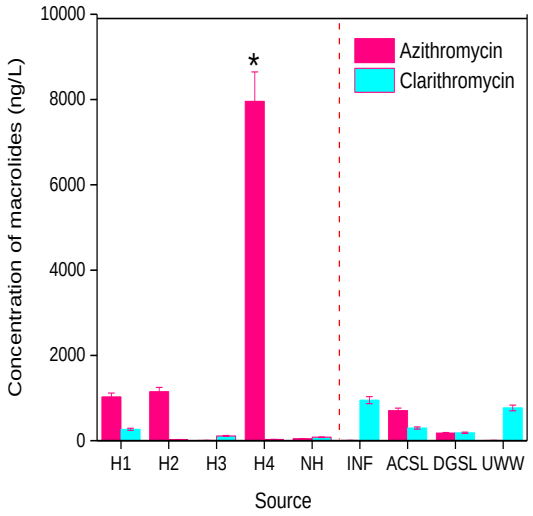
Antimicrobial compounds; AZY, Azithromycin; SDZ, Sulfadiazine; SMZ, Sulfamethoxazole; TRI, Trimethoprim; NOR, Norfloxacin; CIP, Ciprofloxacin; OFL, Ofloxacin; ERY; Erythromycin; CLA, Clarithromycin; TET; Tetracycline; n.d. - not detected; <LOQ-under limit of quantification. **Study sites:** H1-H4: hospital effluent, NH: nursing home, INF: influent, ACSL: activated sludge: DGSL: digested sludge, UWW: urban wastewater.


Antibiotic load per site ng/L
50000
40000
30000
20000
10000
0
TRI
OFL
CIP
NOR
SMZ
SDZ
CLA
AZY
H1
H2
H3
H4
NH
INF
ACSL
DGSL
UWW
Source

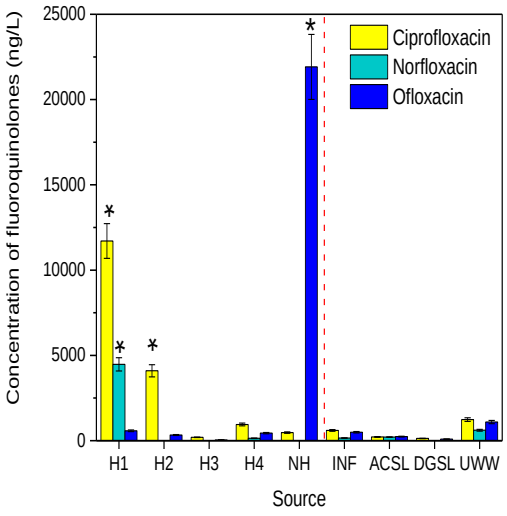
(a)


Total antibiotic concentration ng/L
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Azithromycin
Erythromycin
Clarithromycin
Sulfadiazine
Sulfamethoxazole
Norfloxacin
Ciprofloxacin
Ofloxacin
Trimethoprim
Tetracycline

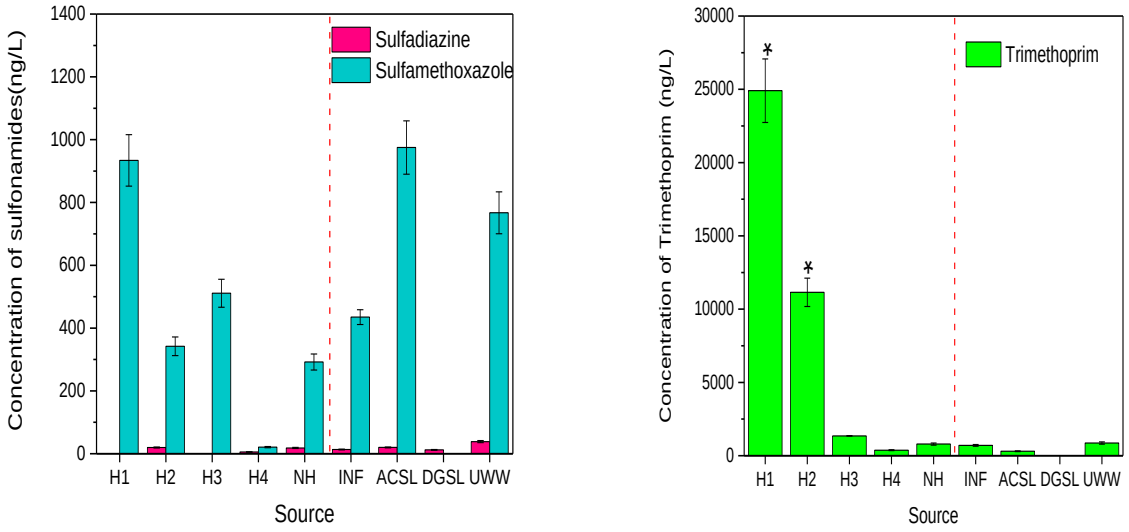
(b)


Concentration of macrolides (ng/L)
10000
8000
6000
4000
2000
0
Azithromycin
Clarithromycin
*
H1
H2
H3
H4
NH
INF
ACSL
DGSL
UWW
Source

(c)


Concentration of fluoroquinolones (ng/L)
25000
20000
15000
10000
5000
0
Ciprofloxacin
Norfloxacin
Ofloxacin
*
H1
H2
H3
H4
NH
INF
ACSL
DGSL
UWW
Source

(d)



(e) (f)

Figure 10. (a) The antibiotics load per location (TRI; trimethoprim, OFL; ofloxacin, CIP; ciprofloxacin, NOR; norfloxacin, SMZ; sulfamethoxazole, SDZ; sulfadiazine, CLA; clarithromycin, AZY; azithromycin) (b**),** the total load for each antibiotic, c d e and f**,** statistical data on the concentrations of the antimicrobial pharmaceutical classes; macrolides, fluoroquinolones, sulfonamides and trimethoprim in the wastewater samples. (*)-asterisk indicates sites with significant concentrations ($P < 0.05$) of the specified antibiotics. **Study sites:** H1-H4: hospital effluents, NH: nursing home, INF: influent, ACSL: activated sludge: DGSL: digested sludge, UWW: urban wastewater.

5.2 Determination of heterotrophic aerobic plate count bacteria and antibiotic resistant Gram-negative bacteria

Heterotrophic aerobic plate count bacteria: The average heterotrophic aerobic plate count for all the sites reached 1.33×10^6 cfu mL^{-1}. It averaged 8.85×10^5 cfu mL^{-1} in hospitals and nursing home effluents and 2.1×10^6 cfu mL^{-1} in the wastewater treatment plant and urban wastewater (**Figure 11***)*. The plate count in the hospital effluents was significantly lower than that in wastewater treatment plant ($P = 0.001$), however, no statistical differences were observed on heterotrophic aerobic plate count within the hospital effluents ($P = 0.200$).

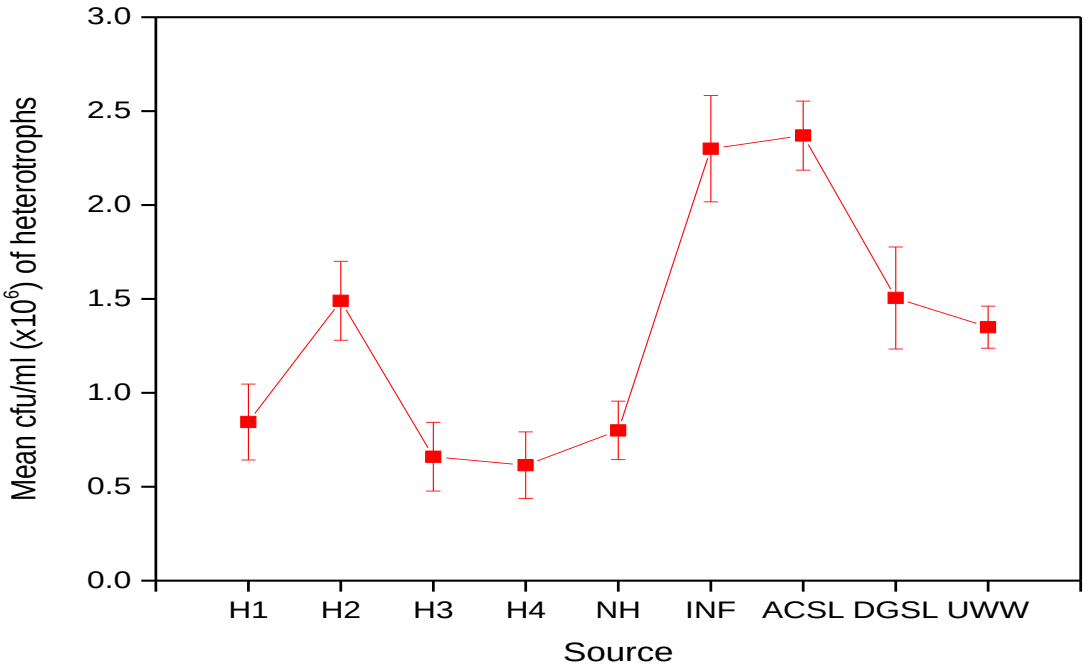


Figure 11. The average heterotrophic live aerobic plate count bacteria expressed as colony forming units per milliliter (cfu ml^{-1}) in the sites. **Study sites:** H1-H4: hospital effluent, NH: nursing home, INF: influent, ACSL: activated sludge: DGSL: digested sludge, UWW: urban wastewater.

Antibiotic resistant Gram-negative bacteria: Bacteria grew in varying numbers on eosin methylene blue agar-ceftriaxone mixture (EMB-CRO) and eosin methylene blue agar-imipenem mixture (EMB-IMP) from all samples (**Table 6**). The average colony forming unit (cfu) count of bacteria growing on EMB-CRO from the hospital and the nursing home effluents was 1.6×10^5, while the cfu count in the wastewater treatment plant and the urban wastewater was 6.8×10^4 cfu mL^{-1}. Although the cfu count was 2-fold higher in the hospital effluents compared to the WWTP and urban wastewater, no significant variation was observed in the cfu count on EMB-CRO among the sites ($p = 0.532$). The average cfu count of bacteria isolated on EMB-IMP from the hospital effluent and the nursing home was 4.3×10^4, while the cfu count in the wastewater treatment plant and the urban wastewater was 5.3×10^3 cfu mL^{-1}. These data show up to 8 times higher loads of bacteria capable of growing on EMB-IMP in hospital effluents and nursing home indicating a significant variation from that of the wastewater treatment plant and urban wastewater ($p = 0.003$).

The total cfu count of the bacteria was significantly higher ($p = 0.0001$) than the cfu count on both EMB-CRO and EMB-IMP.

Table 6. The average concentration of bacteria growing on EMB, EMB-CRO and EMB-IMP from hospital, nursing home, wastewater treatment stages and urban wastewater samples.

	Mean colony counts (cfu mL^{-1}) n =3				
Source	Total cfu count EMB	EMB-CRO	CRO % resistance	EMB-IMP	IMP % resistance
H1	2.1×10^5	9.95×10^4	47.4	4.2×10^4	20
H2	6.65×10^5	3.8×10^5	57	6.2×10^4	9.3
H3	2.6×10^5	1.42×10^5	54.6	7.9×10^4	30.4
H4	2.7×10^5	9.8×10^4	36	2.1×10^4	7
NH	2.9×10^5	1.17×10^5	40.3	1.23×10^4	4.2
INF	1.01×10^5	4.97×10^4	49	3.6×10^3	3.5
ACSL	7.1×10^5	9.8×10^4	13.8	4.6×10^3	0.6
DGSL	2.3×10^5	8.5×10^4	36.9	8.6×10^3	3.7
UWW	3.9×10^5	4.1×10^4	10.5	4.6×10^3	0.1

EMB, Eosin methylene blue agar without antibiotics, EMB-CRO, Eosin methylene blue agar supplemented with ceftriaxone at 2.0 μg/mL, EMB-IPM, Eosin methylene blue agar supplemented with imipenem at 8.0 μg/mL, cfu mL^{-1}, colony forming units per milliliter. % resistance; Ratio of cfu count on EMB-CRO and EMB-IMP expressed as a percentage of total bacterial cfu count on EMB from the hospital effluents (H1-H4), nursing home (NH), wastewater treatment plant (INF-influent, ACSL-activated sludge, DGSL-digested sludge), and urban wastewater (UWW).

The proportion of bacteria growing on EMB-CRO in relation to the total cfu count on EMB varied between 36% (H4) and 57% (H2) in the hospital effluents and the nursing home, and 49% (INF) and 36.9% (DGSL) in the wastewater treatment plant, while it was 10.5% in the urban wastewater.

The proportion of bacteria growing on EMB-IMP in relation to the total cfu count on EMB was much lower than in the case of ceftriaxone. The EMB-IMP/EMB cfu ratios varied between 4.2% (NH) and 30.4% (H3) in the hospital effluents and the nursing home, compared to 3.5% (INF) and 3.7% (DGSL) at the wastewater treatment plant. The lowest prevalence was found in urban wastewater (0.1%). Whereas the EMB-CRO/EMB and EMB-IMP/EMB cfu ratios fluctuated in all the hospital effluent samples from the different sources, the resistance was observed to increase as the treatment progressed from the activated sludge reactor to the digested sludge for both antibiotics.

5.3 Identification of antimicrobial resistant Enterobacterales and *Pseudomonas aeruginosa*

A total of 126 isolates belonging to Enterobacterales were recovered from the samples (**Table S2** and **Table S6**a,b and c). The isolates belonged to *E. coli,* 46% (n = 58), *Klebsiella pneumoniae,* 20.6% (n = 26), *Klebsiella oxytoca,* 13.5% (n = 17), *Enterobacter cloacae,* 7.1% (n = 9), *Citrobacter freundii,* 11.11% (n = 14), *Citrobacter braakii,* 0.8% (n = 1), and *Citrobacter amalonaticus,* 0.8% (n = 1). The isolates were obtained from the following samples: 63.49% (80 strains) from the hospital effluents, 8.7% (11 strains) from nursing home, 20.6% (26 strains) from wastewater treatment plant, and 7.1% (9 strains) from urban wastewater. Other isolates identified as not belonging to the Enterobacterales (*Stenotrophomonas maltophilia,* n = 19, *Elizabethkingia meningoseptica,* n = 7, *Elizabethkingia miricola,* n = 6, and *Acinetobacter junii* n = 1) were not of interest for this study and were excluded from the subsequent analysis. Similarly, a total of 151 *Pseudomonas aeruginosa* isolates were identified from hospital effluents (62.91%, 95 strains), nursing home (7.28%, 11 strains), wastewater treatment plant (24.50%, 37 strains) and urban wastewater (5.29%, 8 strains). Other isolates identified from cetrimid nalidixic agar and excluded from the subsequent analysis were *Aeromonas caviae,* n = 5, *Aeromonas veronii,* n = 7 and *Aeromonas hydrophila,* n = 8).

5.4 Antimicrobial susceptibility profiles and multiple antibiotic resistance indices

The enteric bacteria from the various sites demonstrated variable susceptibility to the tested antibiotics, with isolates from the hospital effluents and the nursing home showing a relatively

higher resistance rate than isolates from the WWTP and the urban wastewater. The multiple antibiotic resistance index (MAR index) for an isolate was calculated as *a/b* where *a* is the number of antibiotics to which an isolate was resistant, and *b* is the total number of antibiotics against which the isolate was tested. The MAR index for a site was calculated as *a/(b*c)* where *a* is the aggregate antibiotics resistance score of all isolates from a sample, *b* is the total number of antibiotics tested and *c* is the number of isolates from sample. Isolates from H3 had the highest resistance rate (MAR index 0.683) among the hospital effluents. Those from the digested sludge were the most resistant (MAR index 0.560) among the wastewater treatment plant isolates, while municipal wastewater had the least resistant isolates (MAR index 0.444) (**Table 7**). *E. coli* demonstrated the highest MAR index (0.65) among the four genera, while *Citrobacter* spp. showed the lowest MAR index (0.39) (**Table 8**). A high prevalence of resistance (>80%) was observed for the third generation cephalosporins (3GCs) ceftriaxone (CRO), ceftazidime (CAZ), cefotaxime (CTX), and cefpodoxime (CPD), while significantly lower resistance rates were measured for carbapenems, imipenem, and meropenem (IMP and MEM) compared to the other antibiotics. From H1 and H2 samples, resistance to IMP was found in 20% and 8% of *Klebsiella* and *E. coli* isolates, respectively, and 1 (4%) *Klebsiella* isolate from H1 was resistant to MEM. Gentamicin (GEN) resistance was the least frequent among the three non-β-lactams.

Table 7. The antimicrobial susceptibility of enteric bacteria in percentage and the MAR indices of the isolates from each site

		Antimicrobial Susceptibility %										
Source	*n* = 126	CRO	CAZ	CTX	CPD	FOX	IMP	MEM	SXT	GEN	CIP	MAR Index
H1	27	100	81	100	100	19	20	4	57	44	78	0.596
H2	25	96	80	96	92	24	8	0	48	60	88	0.592
H3	6	100	100	100	100	50	0	0	100	33	100	0.683
H4	22	96	82	91	91	33	0	0	41	27	68	0.532
NH	11	100	100	92	82	18	0	0	91	27	82	0.591
INF	9	100	100	78	67	22	0	0	56	22	56	0.500
ACSL	12	92	75	83	92	8	0	0	75	18	67	0.508
DGSL	5	100	100	100	100	40	0	0	40	40	40	0.560

UWW	9	100	78	100	100	33	0	0	11	11	22	0.444

Antimicrobial agents; CRO, Ceftriaxone; CAZ, Ceftazidime; CTX, Cefotaxime; CPD, Cefpodoxime; FOX, Cefoxitin; IPM, Imipenem: MEM, Meropenem; SXT, Sulfamethoxazole/trimethoprim; GN, Gentamicin: CIP, Ciprofloxacin, n, number of isolates. **Study sites:** H1-H4: hospital effluent, NH: nursing home, INF: influent. ACSL: activated sludge, DGSL: digested sludge, UWW: urban wastewater.

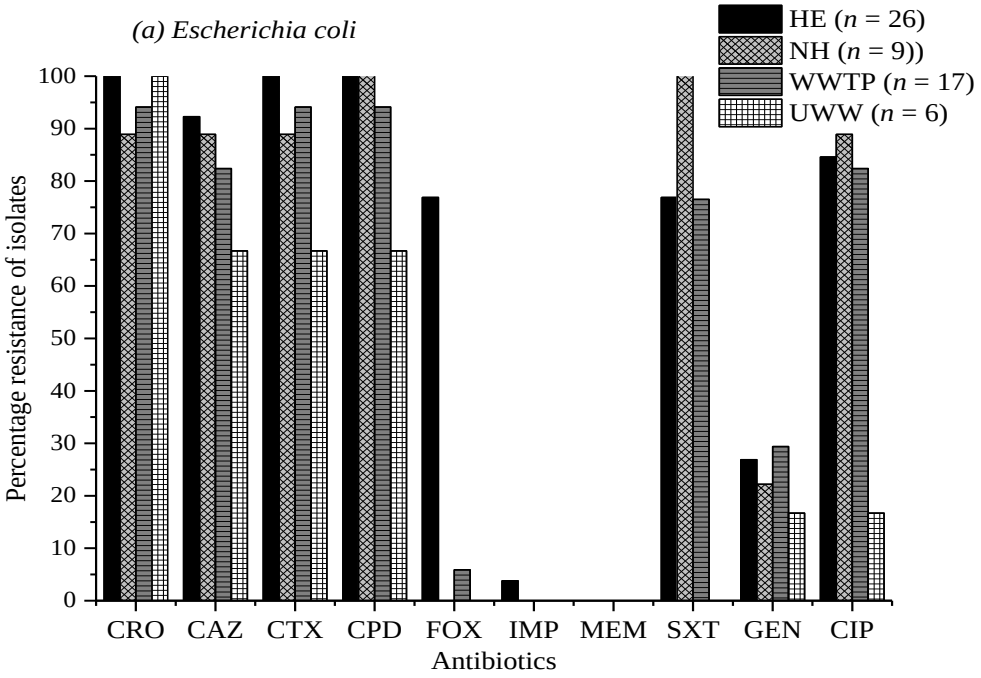
Table 8. Antimicrobial susceptibility of each genus in percentage and their multiple antibiotic resistance indices (n= 126).

Isolates	*E. coli* (*n* = 58)		*Klebsiella* spp (*n* = 43)		*E. cloacae* (*n* = 9)		*Citrobacter* spp (*n* = 16)	
Antimicrobial Agent	R	S	R	S	R	S	R	S
CRO	58 (100)	0 (0)	42 (98)	1 (2)	9 (100)	0 (0)	14 (86)	2 (13)
CAZ	51 (88)	7 (13)	31 (72)	12 (28)	9 (100)	0 (0)	16 (100)	0 (0)
CTX	58 (100)	0 (0)	40 (93)	3 (7)	7 (78)	2 (22)	11 (69.)	5 (31)
CPD	58 (100)	0 (0)	38 (88)	5 (12)	8 (89)	1 (11)	14 (88)	2 (13)
FOX	2 (3)	56 (97)	5 (12)	38 (88)	9 (100)	0 (0)	15 (94)	1 (6)
IMP	2 (3)	56 (97)	6 (14)	37 (86)	0 (0)	9 (100)	0 (0)	16 (100)
MEM	0 (0)	58 (100)	1 (2)	42 (98)	0 (0)	9 (100)	0 (0)	16 (100)
SXT	43 (74)	15 (26)	16 (37)	27 (63)	5 (56)	4 (44)	2 (13)	14 (86)
GEN	15 (26)	43 (74)	24 (56)	19 (44)	0 (0)	9 (100)	5 (31)	11 (69)
CIP	46 79)	12 (21)	29 (67)	14 (33)	5 (56)	4 (44)	7 (44)	9 (56)
MAR index	0.646		0.551		0.555		0.394	

Antimicrobial agents; CRO, Ceftriaxone; CAZ, Ceftazidime; CTX, Cefotaxime; CPD, Cefpodoxime; FOX, Cefoxitin; IPM, Imipenem: MEM, Meropenem; SXT, Sulfamethoxazole/trimethoprim; GN, Gentamicin: CIP, Ciprofloxacin; R, resistant; S, Susceptible, MAR index, multiple antibiotic resistance index.

The resistance rates between β-lactams (ceftriaxone, ceftazidime, cefotaxime, cefpodoxime, cefoxitin, imipenem, and meropenem) and the non-β-lactams (sulfamethoxazole/trimethoprim,

gentamicin, and ciprofloxacin) antibiotics were not significantly different (p = 0.8550). However, a positive correlation was found between resistance in the two groups. Ceftriaxone resistance was positively correlated to SXT and CIP, ceftazidime resistance to SXT, cefotaxime to GEN, SXT and CIP, and cefpodoxime resistance to SXT and CIP. Notably, the *Enterobacter cloacae* and the *Citrobacter* spp. isolates were resistant to cefoxitin (a cephamycin-second generation cephalosporin), unlike the other genera. **Figure 12** a, b, c illustrate the antibiotic resistance patterns of *E. coli*, *K. pneumoniae,* and *K. oxytoca*, *E. cloacae,* and *Citrobacter* species isolates in the hospitals, nursing home, wastewater treatment plant, and municipal wastewater samples.



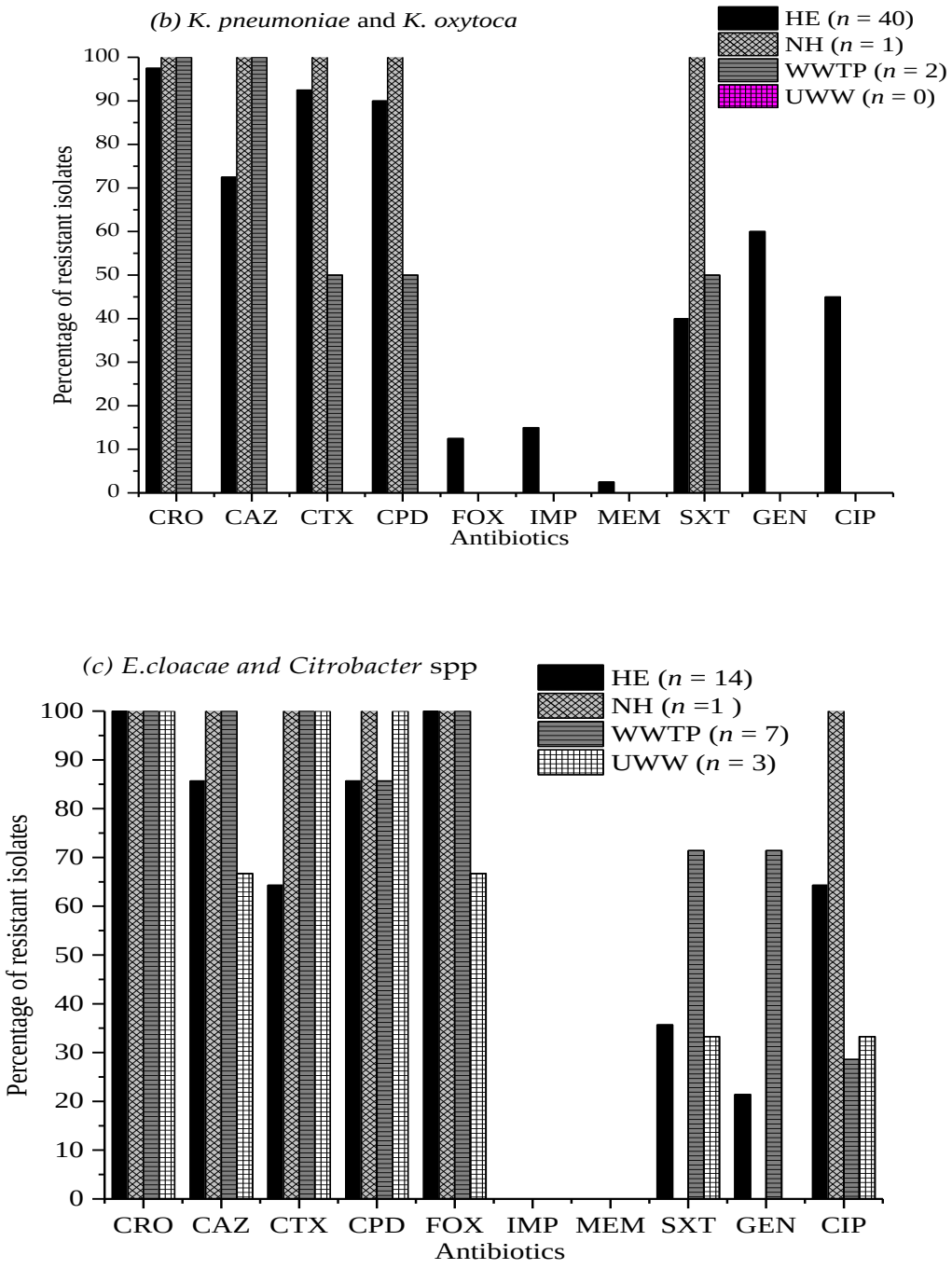


Figure 12. Antimicrobial resistance percentage of (a) *E. coli*, **(**b) *Klebsiella pneumoniae* and *Klebsiella oxytoca* (c) *Enterobacter cloacae,* and *Citrobacter* isolates from four hospitals effluents, nursing home, wastewater treatment plant, and urban wastewater to ten different antibiotics. CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; CPD, cefpodoxime; FOX, cefoxitin; IPM, imipenem: MEM, meropenem; SXT, sulfamethoxazole/trimethoprim; GEN, gentamicin: CIP,

ciprofloxacin. **Study sites;** HE. hospital effluent, NH, nursing home, WWTP, wastewater treatment plant, UWW, urban wastewater. The absence of a bar indicates that no resistance was observed.

5.5 Multiple antimicrobial resistance and co-resistance

Multiresistance (defined as resistance to three or more classes of antibiotics) was observed in 65.08% (*n* = 82) of the isolates. 72.41% (*n* = 42) of *E. coli*, 69.77% (*n* = 43) of *Klebsiella* species, and 40% (*n* = 10) of *Enterobacter* and *Citrobacter* species isolates showed multiple drug resistance (MDR) phenotype, respectively. Although most of the strains were resistant to at least two antibiotic classes, resistance to three and to four chemical classes of antibiotics was observed (**Table 9**). Resistance to four chemical classes was only observed in 11 (19%) *E. coli* isolates, 3 (7%) *K. pneumoniae* isolates, and 2 (13%) *C. freundii* isolates. Four of the isolates resistant to four chemical classes showed resistance to 8 of the 10 antibiotics. The highest rate of multiple drug resistance (≥3) was reported for hospital effluents and nursing home, while the least was observed in urban wastewater. The activated sludge reactor had the highest rate of MDR isolates in the wastewater treatment plant.

Table 9. The number of chemical classes and antibiotics to which the enteric bacterial isolates showed resistance among the 10 antibiotics.

Source	2 classes	MAR (3 classes)	4 classes	7 + antibiotics	10 antibiotics
H1	93% (25/27)	78% (21/27)	0% (0/27)	15 % (4/27)	0% (0/27)
H2	84% (21/25)	84% (21/25)	20% (5/25)	24% (6/25)	0% (0/25)
H3	100% (6/6)	83 % (5/6)	33% (2/6)	50% (3/6)	0% (0/6)
H4	77% (17/22)	46% (10/22)	0% (0/22)	5% (1/22)	0% (0/22)
NH	92% (10/11)	73% (8/11)	27% (3/11)	27% (3/11)	0% (0/11)
INF	56% (5/9)	44% (4/9)	22% (2/9)	11% (1/9)	0% (0/9)
ACSL	75% (9/12)	67% (8/12)	17% (2/12)	17% (2/12)	0% (0/12)
DGSL	40% (2/5)	40% (2/5)	40% (2/5)	40% (2/5)	0% (0/5)
UWW	33% (3/9)	33% (3/9)	0% (0/9)	0% (0/9)	0% (0/9)

Study sites: H1-H4: hospital effluent, NH: nursing home, INFL: influent. ACSL: activated sludge, DGSL: digested sludge, UWW: urban wastewater, MAR, multiple antibiotic resistance. 2 classes refer to either a member(s) of the β-lactams (CRO, CAZ, CTX, CPD, FOX, IMP, and MEM) and one of the non-β-lactams, sulfamethoxazole/trimethoprim (SXT), aminoglycoside (GEN), and fluoroquinolone (CIP). 3 classes; either a member(s) of the β-lactams (CRO, CAZ, CTX, CPD, FOX, IMP, and MEM) and 2 antibiotics belonging to either sulfamethoxazole/trimethoprim (SXT), or aminoglycoside (GEN), or fluoroquinolone (CIP), or the 3 antibiotics belonging to the three non-β-lactam chemical groups (SXT, GEN), CIP). 4 classes; either a member(s) of the β-lactams (CRO, CAZ, CTX, CPD, FOX, IMP, and MEM) and all the other 3 classes, SXT, GEN, and CIP. 7+ antibiotics; more than 7 out of the 10 antibiotics tested. 10 antibiotics; the total number of antibiotics tested (CRO, CAZ, CTX, CPD, FOX, IMP, MEM SXT, GEN and CIP).

The most common associated/co-resistance was found among β-lactams, fluoroquinolone (CIP) and sulfonamide (SXT), while associated resistance to fluoroquinolone (CIP), aminoglycoside (GEN), and sulfonamide (SXT) was less common (**Table 10**). Although co-resistance was common among three chemical antibiotics classes, it also occurred for four chemical classes. Notably, associated resistance against 3GCs, CIP, and SXT was more frequent among *E. coli* and *Klebsiella* isolates. The highest rate of resistance to cephalosporins and fluoroquinolone (CIP) classes was reported in the four hospital samples, while that of cephalosporins and sulfonamide (SXT) occurred in the nursing home effluent samples.

Table 10. The associated/co-resistance to the chemical classes of the antimicrobial agents (n = 126).

3 chemical classes	No.	%	4 chemical classes	No.	%
[β-lactam][CIP][SXT]	58	46	[β-lactam][CIP][GN[[SXT]	16	12.69
[β-lactam][CIP][GN]	38	30.2			
[β-lactam][GN][SXT]	19	15.1			
[CIP][GN][SXT]	13	10.3			

CIP, ciprofloxacin (fluoroquinolone), SXT, sulfamethoxazole, (sulfonamide), GN, gentamicin, (aminoglycoside)

5.6 Phenotypic expression of β-lactamases

Combined disc test of two different antibiotics and their β-lactamase inhibitor combinations were used to classify the isolates as extended-spectrum β-lactamase (ESBL) positive. Cefotaxime/clavulanic acid (CTC 40) and cefpodoxime/clavulanic acid (CD 01) markers defined 87 isolates (69.05%) as ESBL producers (**Figure 13**), (**Table 11**). 62.07% (*n* = 54), 25.3% (*n* = 22), and 6.9% (*n* = 6) of ESBL-positive isolates originated from hospital effluents, wastewater treatment plant, and municipal wastewater, respectively. All *Enterobacter cloacae* isolates were confirmed to be non-ESBL-producing and showed 100% resistance to cefoxitin, which is associated with AmpC cephalosporinase activity. The isolates resistant to imipenem and/or meropenem were confirmed to be metallo-β-lactamase negative by phenotypic test.

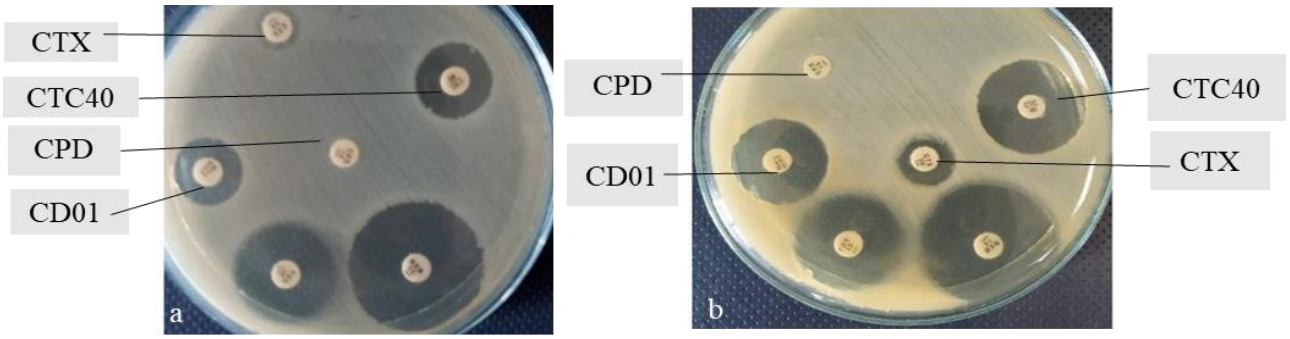


Figure 13. Antibiotic disc assay of (a) *E. coli*, and (b) *K. pneumoniae* showing positive phenotypic test for extended-spectrum β-lactamase production with the combined discs of cefotaxime/clavulanic acid (CTC40) and cefpodoxime/clavulanic acid (CD01)**.**

Table 11. Prevalence of phenotypically expressed extended spectrum β-lactamases (ESBL) based on each genus.

Isolates	ESBL Negative (%) (n = 39)	ESBL Positive (%) (n = 87)	% ESBL Positive/Total Isolates (n = 126)
E. coli (*n* = 58)	8 (14)	50 (86)	40
K. pneumoniae (*n* = 26)	4 (15)	22 (85)	17
K. oxytoca (*n* = 17)	6 (35)	11 (65)	9
E. cloacae (*n* = 9)	9 (100)	0 (0)	0
Citrobacter spp (*n* = 16)	12 (75)	4 (25)	3

5.7 Molecular characterization of ESBL and carbapenemase genes

The ESBL-positive isolates were confirmed to harbor $bla_{\text{CTX-M}}$ (100%) and bla_{TEM} 72.4% (n = 63) with PCR. The *NheI* digestion of the bla_{SHV} PCR product indicating the GLy238 → Ser mutation was positive in 17.2% (n = 15) of the samples (**Table 12**). Additionally, 69% (60 out of 87) of the isolates harbored both $bla_{\text{CTX-M}}$ and bla_{TEM}. This co-occurrence of $bla_{\text{CTX-M}}$ and bla_{TEM} was observed in *E. coli* (62%), *Klebsiella* spp. (49%), and *Citrobacter* spp. (19%). In 17.2% of *Klebsiella* spp. Isolates, both $bla_{\text{CTX-M}}$ and bla_{SHV} genes occurred simultaneously. Furthermore, 11.5% of the total isolates harbored the three groups of β-lactamase genes ($bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV}). The broad-spectrum β-lactamase producers were more widespread in hospital and the nursing home effluents (68.9%) than in wastewater from other sources (WWTP, 24.1%, and urban wastewater, 6.9%). None of the carbapenemase genes bla_{VIM}, bla_{IMP}, bla_{KPC}, $bla_{\text{OXA-48}}$, and bla_{NDM} was detected in the plasmid DNA of the carbapenems-resistant isolates (n = 2, *E. coli*, and n = 7, *Klebsiella* species). However, carbapenems-resistant *K. oxytoca* isolates were shown to carry the bla_{VIM} gene in the genome by a robust colony PCR test. **Figure 14**a-d shows gel images of detected *bla* encoding genes.

Table 12. The number and percentage distribution and co-occurrence of *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ genes among the ESBL positive isolates.

ESBL Gene Family	*E. coli*	*K. pneumoniae*	*K. oxytoca*	*Citrobacter*	Total (% of ESBL Positive, n = 87)
bla$_{CTX-M}$	50 (86)	22 (85)	11 (65)	4 (25)	87 (100)
bla$_{TEM}$	39 (67)	16 (62)	5 (29)	3 (19)	63 (72)
bla$_{SHV}$	0 (0)	14 (54)	1 (6)	0 (0)	15 (17)
bla$_{CTX-M}$ + *bla*$_{TEM}$	36 (62)	16 (62)	5 (29)	3 (19)	60 (69)
bla$_{CTX-M}$ + *bla*$_{SHV}$	0 (0)	14 (54)	1(6)	0(0)	15 (17)
bla$_{CTX-M}$ + *bla*$_{TEM}$ + *bla*$_{SHV}$	0 (0)	10 (38)	0 (0)	0 (0)	10 (11)
Total number (ESBL positive and ESBL negative)	58	26	17	16	126

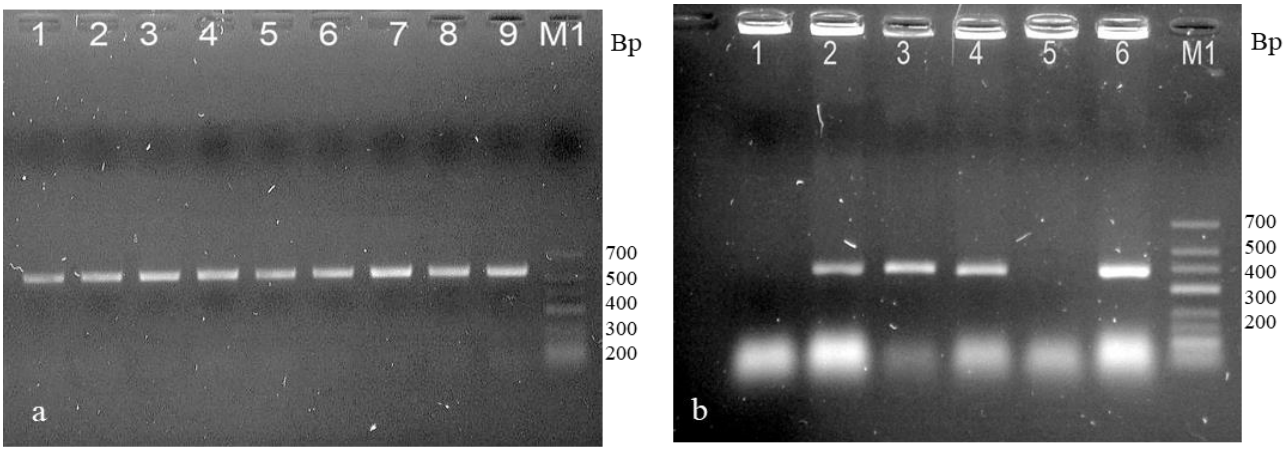
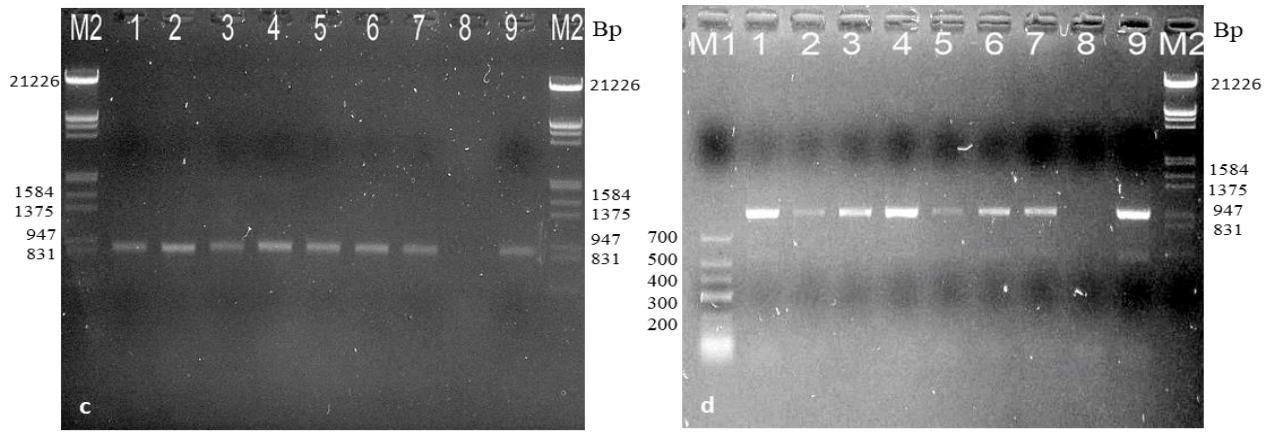


Figure 14. a-d. Gel images of *E. coli* and *Klebsiella* spp. showing the β-lactamase genes, *bla*$_{CTX-M}$, *bla*$_{VIM}$, *bla*$_{SHV}$, and *bla*$_{TEM}$. (a) Lanes 1- 4, *E. coli*, Lanes 5 and 6 *K. pneumoniae*, Lanes 7 and 8 *K. oxytoca*, all strains carrying plasmid borne *bla*$_{CTX-M}$ gene with product size 544 bp. Lane 9, positive control strain *K. pneumoniae* 722, and (b). Lanes 1 and 5 *K. oxytoca* strains with missing *bla*$_{VIM}$ gene, Lanes 2, 3, and 4, *K. oxytoca* strains carrying chromosomally borne *bla*$_{VIM}$ gene demonstrated by colony PCR, product size 390 bp, Lane 6, positive control strain *K. pneumoniae* 1745, M1, Low range ladder molecular weight marker (700bp).



(c) Lanes 1 - 7, *K. pneumoniae* spp carrying plasmid borne bla_{SHV} gene (*Nhe*I digested), product size 865 bp, lane 8 distilled water, lane 9, positive control strain, *K. pneumoniae* MGH 78578, (d). Lanes 1- 4, *E. coli*, lanes 5 -7, *Klebsiella* spp carrying plasmid borne bla_{TEM} gene, product size 963 bp. lane 8, *Klebsiella* spp lacking bla_{TEM} gene, Lane 9, positive control strain *K. pneumoniae* 722, M2, Lambda phage DNA- *Eco*RI/*Hind*III molecular weight marker (21226 bp), M1, Low range ladder molecular weight marker (700 bp).

5.8 Next generation sequencing of plasmids

Selected isolates belonging to *E. coli* (n = 10), *Klebsiella* spp. (n = 10), and *Citrobacter* spp. (n = 1) were subjected to next generation sequencing and de novo assembly of plasmid sequences. Number, total length, and N50 of assembled contigs ranged between 17 and 64, 182 477–547 810 bp, and 11 403–62 343 bp, respectively **(Table S4**). According to maximal unique and exact match (MUM) indices the isolates were clustered into six groups, designated as G1–G6 (**Figure 15**). The two main groups contained the majority of *E. coli* (G1) and *K. pneumoniae* (G2) isolates and the four minor groups (G3–G6) enclosed single, unrelated isolates. Cluster G1 could be subdivided into 3 subgroups (G1-a, G1-b, and G1-c).

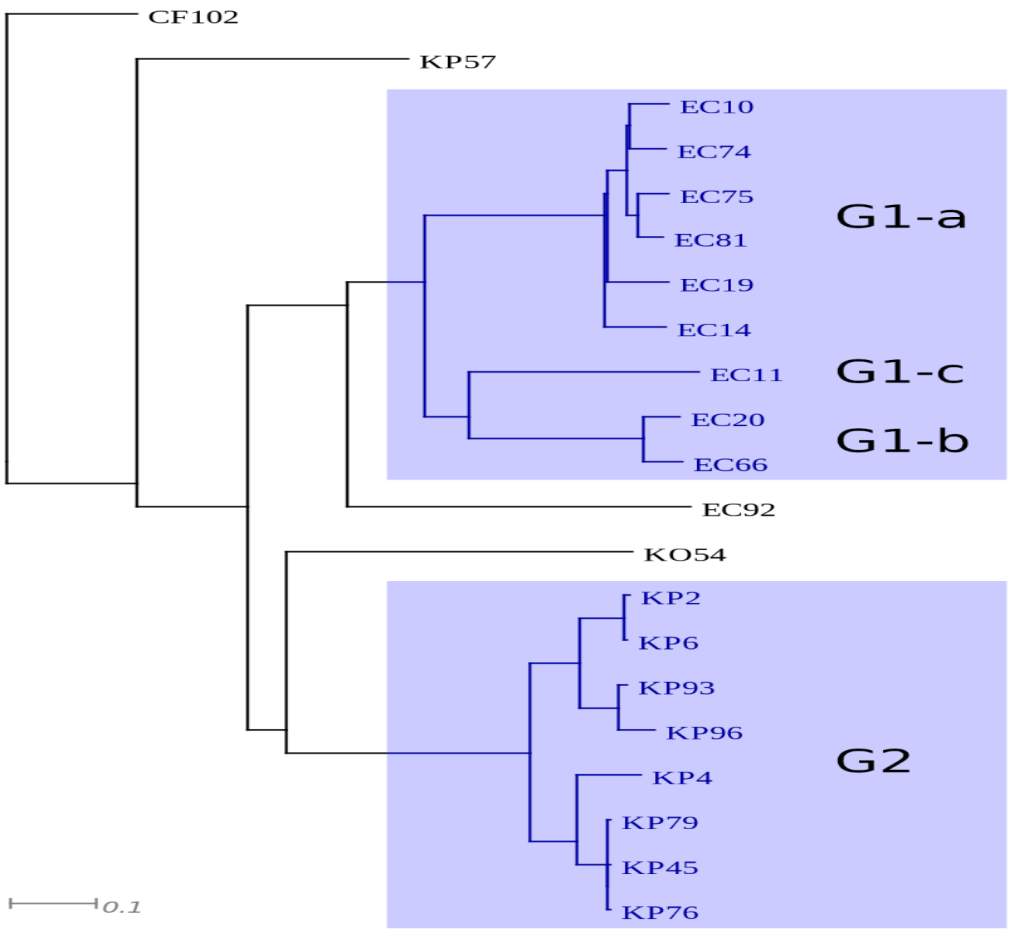


Figure 15. Representation of clusters based on MUMi distance of plasmid sequences. The major clusters are highlighted with blue.

All isolates (n = 9) of G1 were identified as *E. coli* and were shown to harbor $bla_{\text{CTX-M-27}}$ type ESBL gene, aminogylcoside (*aadA5*, *aph(3")-Ib*, *aph(6)-Id*), folate inhibitor (*dfA17*, *sul1*, *sul2*), tetracycline (*tet(A)*), macrolide (*mph(A)*), and quaternary ammonium compound (*qacE*Δ) resistance genes. The *dfrA17*, *aadA5*, *qacE*Δ, and *sul1* genes were part of a class I integron. The integron and the *mph(A)* gene were co-located on identical contigs in each isolate. Besides, *aph(6)-Id*, *aph(3")-Ib*, and *sul2* were also found to be co-localized in all isolates, but the contigs carrying them differed according to the subgroups (**Figure 16**a-c).

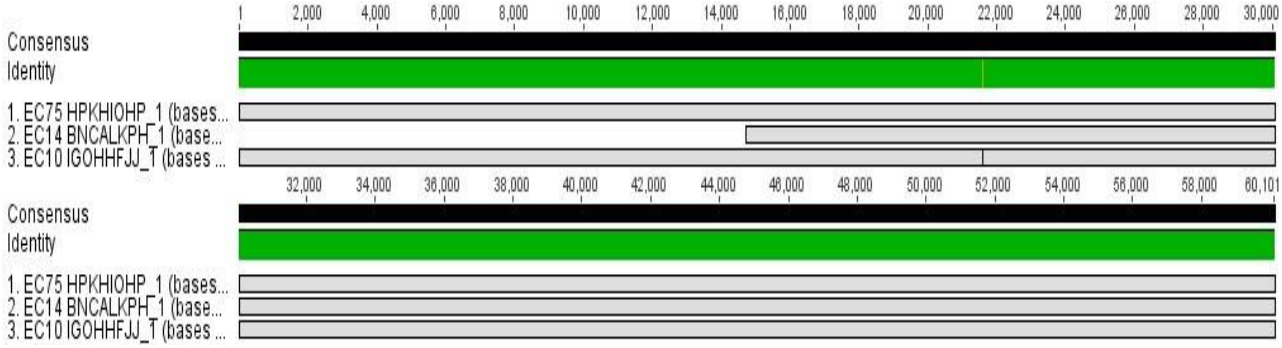


Figure 16 (a). Mauve alignment of contigs in EC10, EC14 and EC75 carrying *floR*, *aph(6)-Id*, *Tn*2 (*tnpA*, *tnpR*, *bla*$_{\text{TEM-1}}$), *aph(3'')-Ib* and *sul2*.

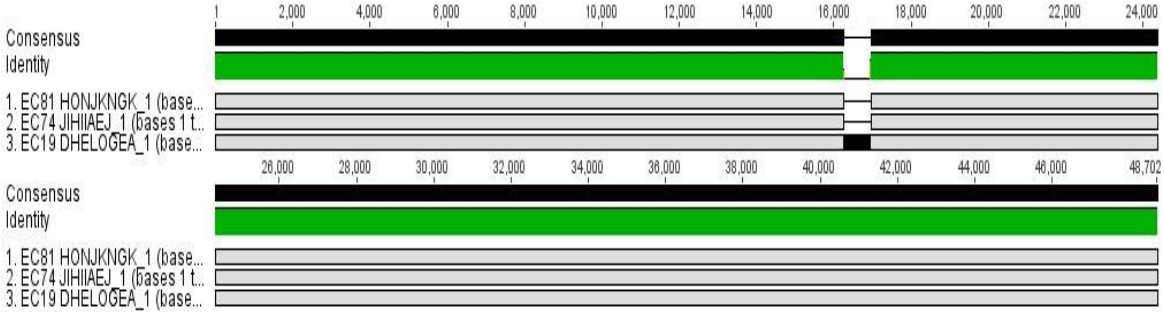


Figure 16 (b). Mauve alignment of contigs in EC19, EC74 and EC81 carrying *tet(A)*, *aph(6)-Id*, *Tn*2 (*tnpA*, *tnpR*, *bla*$_{\text{TEM-1}}$), *aph(3'')-Ib* and *sul2*

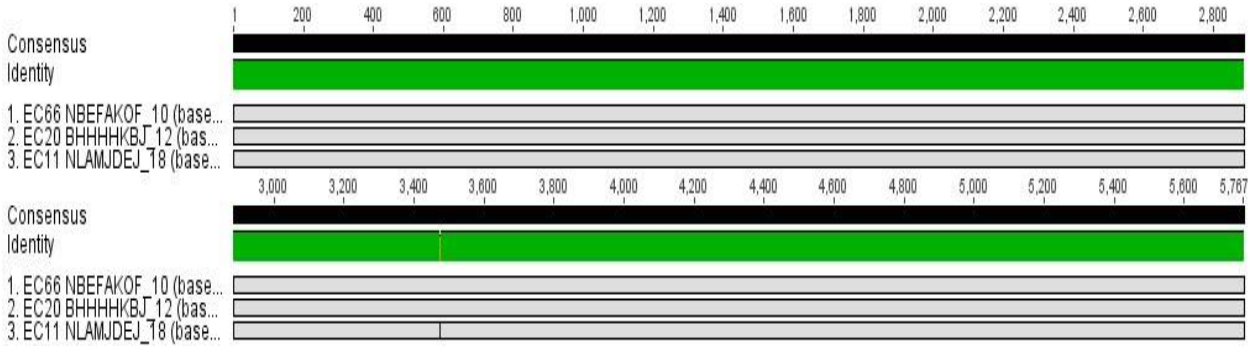


Figure 16 (c). Mauve alignment of contigs in EC11, EC20 and EC66 carrying *tet(A)*, *aph(6)-Id*, *aph(3'')-Ib* and *sul2*

In subgroup G1-a (**Figure 16**a–b), a *Tn*2 transposon carrying *bla*$_{\text{TEM-1}}$ was inserted between *aph(6)-Id* and *aph(3″)-Ib* and either *floR* or *tet(A)* was located upstream from this genetic structure. In subgroup G1b-G1c (**Figure 16**c) *tet(A)* was located upstream from *aph(6)-Id, aph(3″)-Ib,* and *sul2*. The presence of *floR* and *tet(A)* was shown for subgroup G-1a and all subgroups of G1, respectively. Different plasmid incompatibility groups were characteristic for the three subgroups: in subgroup G1-a IncB/O/K/Z, IncFIA, IncFIB, and IncFII, in subgroup G1-b IncFIA, IncFII, and IncI, and in subgroup G1-c IncFIA, IncFIB, IncFII, and IncI were detected (**Table S4**).

Cluster G2 enclosed eight, closely related *K. pneumoniae* isolates. The presence of multiple β-lactamase genes (ESBL: *bla*$_{\text{CTX-M-27}}$, non-ESBL: *bla*$_{\text{TEM-1}}$, *bla*$_{\text{OXA-1}}$), aminoglycoside (*aac(6')-Ib-cr*, *aph(3″)-Ib,* and *aph(6)-Id*), chloramphenicol (*catB3*), folate inhibitor (*dfrA14*, *sul2*), and quinolone (*qnrB1, aac(6')-Ib-cr*) resistance genes were characteristic for all isolates of G2. The *dfrA14* was carried by a class I integron which lacked the 3′ conserved sequence. The contigs carrying the aforementioned resistance genes were highly identical in all isolates (Table S2). Additionally, three isolates also harbored *aac(3)-IIa* on identical contigs. Two plasmid incompatibility groups (IncFIB and IncFII) were identified in this cluster (**Table S4**).

The four unrelated isolates, namely *C. freundii* CF102, *K. pneumoniae* KP57, *K. oxytoca* KO54, and *E. coli* EC92, were shown to harbor either *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV-12}}$, *bla*$_{\text{CTX-M-30}}$, or *bla*$_{\text{CTX-M-1}}$ ESBL genes, respectively. Besides, a diversity in plasmid incompatibility groups and multiple antibiotic resistance genes (Table S1) were identified in these isolates, except for EC92 in which only the ESBL gene was detected. Genes *aac(6')-Ib*, *aadA1*, *bla*$_{\text{OXA-9}}$, and *bla*$_{\text{TEM-1}}$ were located on a transposon (*Tn*1331) in KP57 and KO54. In addition to *Tn*1331, KP57 also harbored *aph(3″)-Ib* and *aph(6)-Id* aminoglycoside resistance genes. In CF102 *aac(3)-IIa*, *bla*$_{\text{OXA-1}}$, *catB3*, and *aac(3)-IIa* were identified on contigs that were highly similar to those found in *K. pneumoniae* isolates of G2. Moreover, CF102 also carried *aph(3″)-Ib*, *cmlB1*, and *tet(A)* genes as well.

5.9 Characterization of *Pseudomonas aeruginosa*

The 151 isolates of *P. aeruginosa* showed variable susceptibilities to the five antibiotics tested. The MAR index for *P. aeruginosa* was 0.8. 97.4% (147 strains exhibited MAR index higher than 0.2. Only 4 (2.6%) isolates had a MAR index of 0.2. Isolates from H4 and NH demonstrated the highest MAR index (0.85) among the samples from hospitals and the nursing home while those from the digested sludge had a higher MAR index (0.88) among the WWTP samples (**Table 13**). Gentamicin demonstrated the highest levels of resistance among the five antibiotics tested on *P. aeruginosa* (92.72%, 140 strains), while ceftazidime resistance was the highest of the three β-lactams (85.43%, 129 strains) and the isolates showed the least resistance to meropenem (57.62%, 87 strains) (**Table 14**). A positive correlation was found where the concentrations of both ciprofloxacin and ofloxacin residues in the wastewater samples resulted in an increase in the resistance phenotype observed in ciprofloxacin among the *P. aeruginosa* isolates (r - 0.015, p = 0.940 and r - 0.003, p = 0.989, respectively).

Table 13. The antimicrobial susceptibility in percentage and the multiple antibiotic resistance indices of *P. aeruginosa* in each site.

		Antimicrobial susceptibility %					
Source	n = 151	CAZ	MEM	IMP	GN	CIP	MAR index
H1	35	80	77	97	89	69	0.77
H2	21	90	43	100	100	90	0.82
H3	12	92	17	100	100	50	0.7
H4	27	89	78	96	96	85	0.85
NH	11	91	64	91	100	73	0.85
INF	12	67	42	83	92	50	0.68
ACSL	16	94	56	88	94	88	0.84
DGSL	9	89	78	78	89	100	0.88
UWW	8	75	0	25	38	38	0.4

Antimicrobial agents; CAZ, ceftazidime; MEM, meropenem; IPM, imipenem: GN, gentamicin: CIP, ciprofloxacin. **Study sites:** H1-H4: hospital effluent, NH: nursing home, INF: influent. ACSL: activated sludge, DGSL: digested sludge: UWW: urban wastewater, MAR, multiple antibiotic resistance index.

Table 14. Antimicrobial susceptibility of *P. aeruginosa* from wastewater (n = 151).

Antimicrobial agents	Resistant (No. %)	Susceptible (No. %)
Ceftazidime	129 (85)	22 (14)
Meropenem	87 (58)	64 (42)
Imipenem	126 (83)	25 (17)
Gentamicin	140 (93)	11 (7)
Ciprofloxacin	113 (75)	38 (25)

Multiple drug resistance (MDR) in *P. aeruginosa* was observed in 72.85% (110) of the isolates that exhibited resistance to the three antibiotic classes (beta-lactams, aminoglycoside, and fluoroquinolone). MDR in hospital effluents and nursing home was 72.6% (77/106) while it was 64.86% (24/37) in the wastewater treatment plant and 25% (2/8) in the urban wastewater. H2 and H4 demonstrated the highest MDR (95.2% and 88.9%) while H3 recorded the lowest multidrug resistant isolates (19%) (**Table 15**). In the wastewater treatment plant, the isolates from the digested sludge demonstrated the highest MDR 77.8% (7/9). Except for H3 and urban wastewater, all the other samples recorded a number of isolates showing resistance to all five antibiotics. An associated resistance occurred more frequently between β-lactams and aminoglycoside (gentamicin), 21.9% (33/151) compared to β-lactams and fluoroquinolone (ciprofloxacin) 4.6% (7/151).

Table 15. Number of chemical classes resisted by *P. aeruginosa* species and percentage of isolates showing resistance to the 5 antibiotics.

Source	2 classes	MDR (3 classes)	all 5 antibiotics
H1	97% (34/35)	60% (21/35)	34% (12/35)
H2	100% (21/21)	95% (20/21)	38% (8/21)
H3	100% (12/12)	19% (4/12)	0% (0/12)
H4	100% (27/27)	89% (24/27)	82% (22/27)
NH	100% (11/11)	73% (8/11)	46% (5/11)
INF	92% (11/12)	42% (5/12)	8% (1/12)
ACSL	100% (16/16)	75% (12/16)	25% (4/16)
DGSL	100% (9/9)	78% (7/9)	56% (5/9)
UWW	38% (3/8)	25% (2/8)	0% (0/8)

Study sites; H1-H4: hospital effluent, NH: nursing home, INF: influent. ACSL: activated sludge, DGSL: digested sludge: UWW: urban wastewater, 2 classes; any 2 of the 3 classes β-lactams, fluoroquinolone and aminoglycoside: MDR, multiple drug resistance; all the 3 classes β-lactams, fluoroquinolone and aminoglycoside. 5 antibiotics; ceftazidime, meropenem, imipenem, gentamicin, and ciprofloxacin.

5.10 Phenotypic expression of β-lactamases

The combined disc test of ceftazidime/clavulanic acid (CZC 20) confirmed 19.3% (25/129) ESBL positive strains out of the total ceftazidime resistant isolates, whereas IPM/EDTA (IEL 292) and MEM/EDTA (MEL 292) defined 44% (56/126) and 66% (57/87), respectively, MBL positive strains out of the carbapenem-resistant isolates (**Figure 16**). 80%, (20/25) of ESBL and 68%, (39/57) of MBL positive strains originated from the hospital effluents and nursing home, whereas 20% (5/25) of ESBL and 29.8% (17/57) of MBL positive strains were recovered from the WWTP and 4% (1/25) of ESBL positive strains were from the urban wastewater.

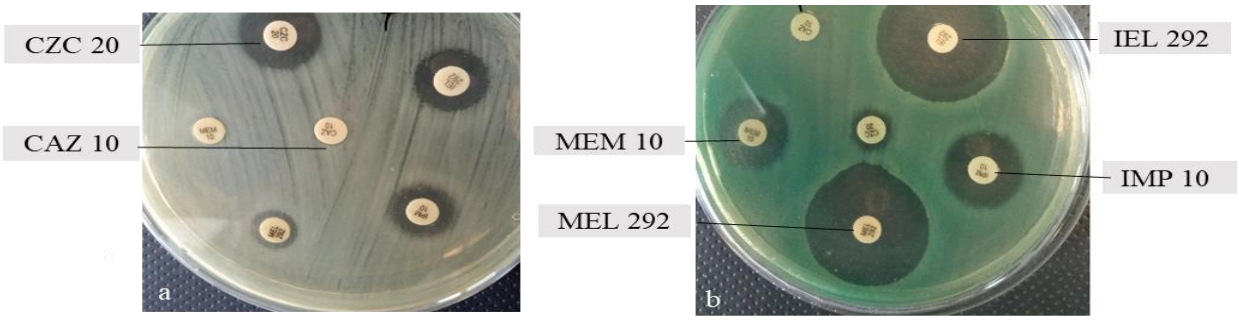


Figure 16. Antibiotic disc assay of *P. aeruginosa* isolates showing (a) Positive phenotypic test for ESBL production with ceftazidime (10 µg) and ceftazidime/clavulanic acid (CZC 20) and (b) expressing MBL production with imipenem (10 µg) and meropenem (10 µg) and the combined discs imipenem/EDTA (IEL 292) and meropenem/EDTA (MEL 292).

Whereas none of the ESBL positive isolates carried the *bla*$_{SHV}$ gene, two of them were each confirmed to harbor both *bla*$_{CTX-M}$ and *bla*$_{TEM}$, while 4% (1/25) carried both *bla*$_{CTX-M}$ and *bla*$_{TEM}$. The metallo-β-lactamase positive isolates carried *bla*$_{VIM}$ gene (100%) while the *bla*$_{NDM}$ gene occurred in 1.8% (1/57) of the isolates. None of the isolates was positive for *bla*$_{IMP}$ and *bla*$_{SPM}$ genes. **Figure 17**a and b show gel images of *Pseudomonas* isolates carrying carbapenamases *bla*$_{NDM}$ and *bla*$_{VIM}$ genes whereas **Figure 18** shows gel images of the two portions of the VIM gene amplified for sequencing. Metallo-β-lactamase producers were more widespread in hospital effluents than in other wastewater sources (82%, hospital and nursing home effluents, 12%, WWTP and 5%, urban wastewater).

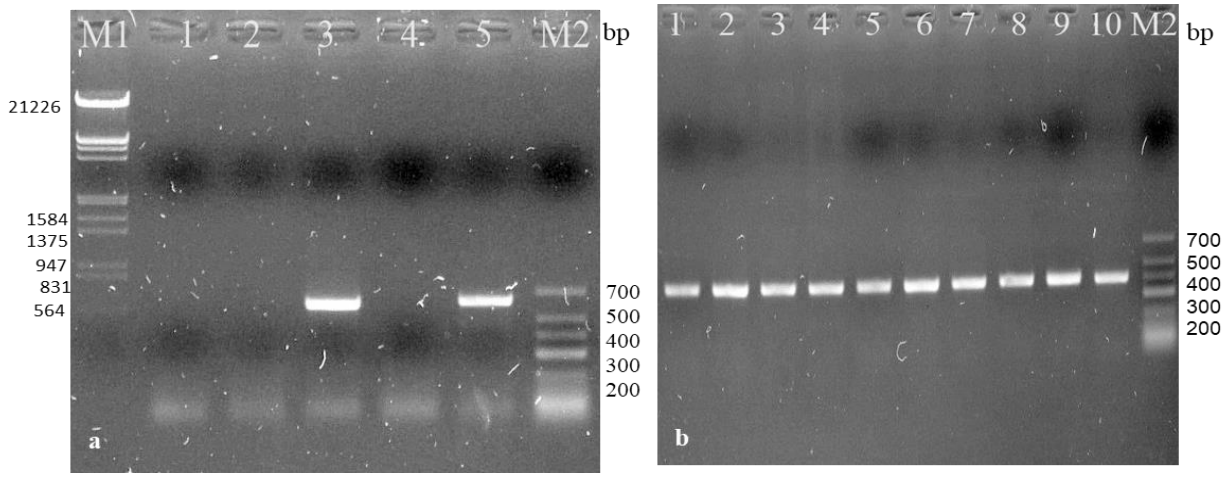


Figure 17. Genomic DNA of *P. aeruginosa* spp, carrying *bla*$_{NDM}$ gene and *bla*$_{VIM}$ gene. (a) Lane 3, Genomic DNA of *P. aeruginosa* carrying *bla*$_{NDM}$ gene, product size 621 bp, lanes 1, 2, and 4, *P. aeruginosa* lacking the gene, lane 5, *Klebsiella pneumoniae* 131946 positive control strain, (b) Lanes 1-9, Genomic DNA of *P. aeruginosa* carrying *bla*$_{VIM}$ gene, product size 390 bp, lane 10, *Klebsiella pneumoniae* 1745 positive control strain, M1, Lambda phage DNA- *EcoRI/HindIII* (21226 bp), M2, Low range ladder (700 bp)- molecular weight markers.

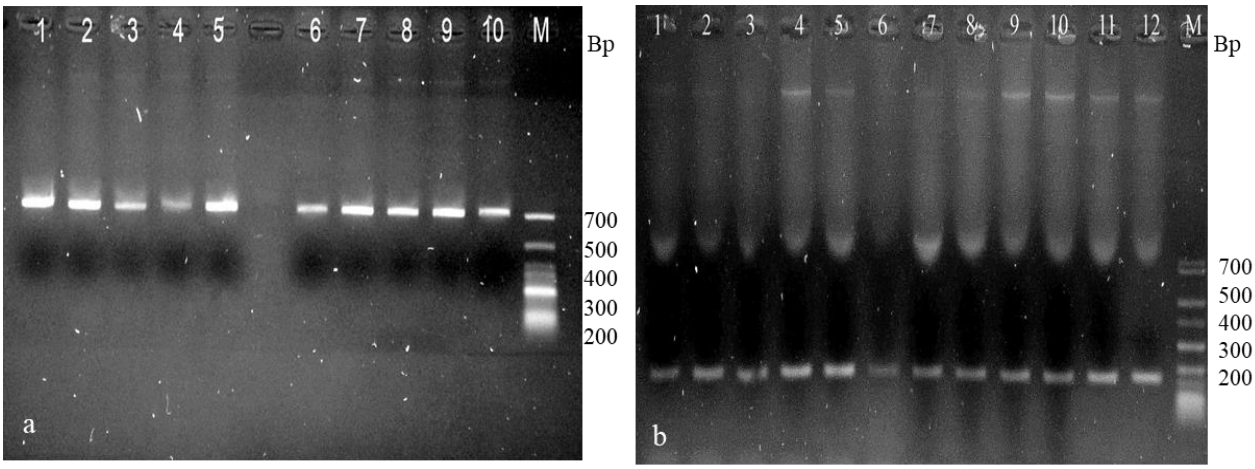


Figure 18. Genomic DNA of *P. aeruginosa* showing the two portions of *bla*$_{VIM}$ gene used for sequencing. (a) Lanes 1-9, Genomic DNA of *P. aeruginosa* carrying *bla*$_{VIM}$ gene, product size 775 bp, lane 10, *Klebsiella pneumoniae* 1745 positive control strain, (b) Lanes 1-11, Genomic DNA of *P. aeruginosa* carrying *bla*$_{VIM}$ gene, product size 161 bp, lane 12, *Klebsiella pneumoniae* 1745 positive control strain M, Low range ladder (700 bp), molecular weight marker.

5.11 Sequences analysis of the VIM gene

The Sanger sequence alignment analysis showed identity to *P. aeruginosa* class 1 metallo-beta-lactamase (*bla*$_{VIM}$-$_{4}$) sequence without substitution at any known nucleotide sequences in all the 33 samples investigated for the VIM gene **Table 16**.

Table 16. The percentage sequence identity of selected *P. aeruginosa* isolates to *P. aeruginosa* class 1 metallo-beta-lactamase (*bla*$_{VIM\text{-}4}$) reference gene.

Sample	Identity %	length (bp)
12	96	37-792
13	97	35-786
14	99	35-793
15	99	53-792
16	90	53-793
17	98	35-793
18	98	35-792
19	99	37-793
20	98	35-793
21	98	35-793
22	99	39-790
23	99	53-793
24	99	35-773
25	99	37-793
26	98	35-793
27	98	39-792
28	98	35-782
29	98	37-793
30	99	37-793
31	99	35-792
32	99	37-791
33	99	39-793

6.0 DISCUSSION

The occurrence of antibiotics and/or their metabolites in wastewater is a subject of concern due to their potential ecological risks and their role in the evolution of antibiotic resistant bacteria (WHO 2014, Berendonk et al. 2015, Kumar et al. 2019). Although antibiotics are naturally produced by microorganisms as weapons for survival in their habitats, anthropogenic activity leading to their discharge via wastewater is considered the major source of environmental contamination (Felis et al. 2020). Analysis of the samples in this study demonstrated the presence of various antimicrobial pharmaceuticals embedded in the wastewater at varying concentrations from all the sources. The study reported the occurrence of the antimicrobial classes macrolides, sulfonamides, trimethoprim, and fluoroquinolones (ngL^{-1}), which are the most detected substances from the environmental matrices because of their ability to persist in the aqueous environment besides being the most frequently prescribed classes in veterinary and human medicine (Wang and Wang 2016, Korzeniewska and Harnisz 2020). The data presented demonstrates that usage of these antibiotics occurs at a higher frequency in the healthcare facilities than in the community as demonstrated by the higher antibiotics loads in the samples obtained from the hospitals and the nursing home. Hospital wastewaters contribute significantly to the environmental burden of antibiotics, which drive the emergence of antibiotic resistance (Kummerer 2004, Zhang et al. 2009c, Davies and Davies 2010, Harris et al. 2012). Trimethoprim concentration was the highest detected in this study, especially in hospital samples and studies have shown that, in hospital effluents, it has been detected at a concentration less than 3 μgL^{-1} and in almost all wastewater samples in which this compound has been determined, it occurred in the range of 100 – 6000 ngL^{-1}, with its concentration in WWTP effluents reported in the range of 60–3000 ngL^{-1} (Santos et al. 2013, Mendoza et al. 2015). The occurrence of trimethoprim content above 1.5 μgL^{-1} has been demonstrated in WWTP influents in Sweden, Italy, Croatia, and Greece (Verlicchi et al. 2012, Kosma et al. 2014). The sulfonamide; sulfamethoxazole which is the most commonly used drug in this class occurred in all the samples at a concentration higher than that of sulfadiazine. In Italy, Portugal and Sweden, levels of sulfamethoxazole reaching up to 6.5 μgL^{-1} , 8.7 μgL−1, and 13 μgL^{-1}, respectively were detected in hospital effluents (Lindberg et al. 2004, Verlicchi et al. 2012, Santos et al. 2013). The concentration of sulfonamides in WWTP influents and effluents was found to range from tens to hundreds of ng L^{-1}, which is attributed to consumption of these agents in the community sector

(Golovko et al. 2014, Papageorgiou et al. 2016). A study done in a German WWTP found sulfamethoxazole in the influents and effluents at a concentration of up to 2 μg L^{-1} (Rossmann et al. 2014) whereas in a WWTP in Portugal, a concentration reaching up to a maximum of 1.7 μg L^{-1} has been determined (Santos et al. 2013). Among the macrolides, azithromycin concentration was higher in the hospital effluents while clarithromycin occurred at a much higher concentration in wastewater samples. The occurrence of these antibiotics in wastewater is of environmental significance due to their potential risks, which necessitated their inclusion in the EU Commission watch list of pollutants of concern (EU Decision, 2015/495 of March 20, 2015). Hospital effluents have been reported as the main input routes for these compounds into the natural environment, especially, WWTP where the concentration of some, particularly clarithromycin and azithromycin, which are the most frequently detected ones in influent has been found to be high (Verlicchi et al. 2012, Loos et al. 2018), even though azithromycin was not reported in our samples. Quantities of macrolides ranging between 1 and 10 μg L^{-1} have been found in hospital effluents (Gros et al. 2013, Santos et al. 2013). Fluoroquinolones are among the most frequently used antibiotics and their consumption in human medicine is estimated to account for 7% of the total antimicrobials consumption (Szymańska et al. 2019). Their occurrence in different aqueous environmental matrices has been reported with the maximum concentrations typically occurring in hospital effluents and WWTP influents. Their mobility in the aquatic environment due to their high hydrophilic property contributes to their occurrence in both groundwater and drinking water samples (Hanna et al. 2018, Reis et al. 2019). Their rapid spread in the environment with potential ecotoxicological effects necessitated the inclusion of ciprofloxacin in the EU Commission watch list (EU Decision, 2018/840 of June 5, 2018) (Felis et al. 2020). Ciprofloxacin and ofloxacin appear to be the dominant ones with high detection frequency and high concentration of up to 4600 ng L^{-1}, ciprofloxacin and 7870 ng L^{-1} ofloxacin in wastewater detected by Lindberg et al. (Lindberg et al. 2007) which is consisted with the findings of this study where ciprofloxacin and ofloxacin were detected in the samples at a relatively high concentration. Tetracyclines are highly hydrophobic with low volatility, making them very stable in the aquatic environment, and are commonly detected in WWTPs due to their widespread use in aquaculture and animal husbandry (Daghrir and Drogui 2013). However, tetracycline was below the limit of quantification or was not detected in our samples possibly due to the absence of intensive animal husbandry within the study area since tetracyclines are commonly used in animals. The significantly high heterotrophic aerobic plate

count in the influent and activated sludge was attributed to the high levels of organic loading resulting in high biomass characterized by these stages of wastewater treatment. It is notable that the cfu count of heterotrophic aerobic plate count from the urban wastewater was higher than that from three of the hospitals (H1, H3 and H4) and the retirement home, which is indicative of higher organic loading of wastewater from the urban communities.

The annual antibiotics consumption in Europe is estimated to be approximately 10 000 tons with half used in human medicine and half in veterinary medicine and almost 26% of the antimicrobial agents are administered in hospitals (Penalva et al. 2019). Hungary ranks among the countries with the lowest antimicrobial drug consumption rate (defined daily dose per 1000 inhabitants per day) both in the hospitals and the community sector in the European union/European Economic area based on the annual European Surveillance of Antimicrobial Consumption Network (ESAC-Net) report (ECDC 2020). Despite the low consumption, disposal of untreated hospital effluents containing these antimicrobials or their metabolites may have selected for the development of antibiotic resistant bacteria based on our findings. This study indicated a remarkable concentration of enteric bacteria resistant to extended-spectrum cephalosporins in hospital effluents, nursing home and the WWTP with bacterial cfu count being 2 fold higher in hospital effluents and nursing home than in the WWTP and the urban wastewater. Although hospital wastewater has been described as a highly selective environment that may contribute to the maintenance of resistant bacteria discharged into the natural environment (Prado et al. 2008, Yang et al. 2009), they are not necessarily the primary source of resistant bacteria in municipal wastewater since they contribute less than 2% of the total volume of the wastewater (Carraro et al. 2016), thus other possible sources such as agricultural runoffs require to be monitored (Lettieri Teresa et al. 2018).

Our data on β-lactam resistant bacteria indicate the likelihood of bacteria of fecal origin carrying resistance traits from the source population being present in hospital effluents discharged into the wastewater network. This finding is consistent with a similar observation involving cephalosporin resistance in wastewater (Czekalski et al. 2012). Other studies have found an increase in antibiotic resistant bacteria in hospital wastewater networks which has been attributed to large-scale antimicrobial usage in the hospital setting and the presence of their residues, especially at sub-inhibitory concentrations over extended periods (Morris et al. 2008, Chartier 2014). In a related study, cephalosporin resistant bacteria were also more concentrated in hospital wastewater

compared to WWTPs (Aali et al. 2014). Although bacterial resistance to imipenem (another β-lactam) was very low across our study locations, a significant increase in its resistance rate was observed in hospital effluents relative to the WWTP. However, this was not attributed solely to the members of Enterobacterales but rather due to the presence of other Gram-negative bacteria, most notably non-fermenting *Stenotrophomonas maltophila,* which was frequently detected in the hospital samples and possesses an intrinsic resistance to imipenem.

We observed a more or less similar rate of resistance among the Enterobacterales across the hospital and the nursing home effluents, measured by the multiple antibiotic resistance indices (MAR index), despite a huge variation in the bed capacity. H2 and H3, with the lowest bed capacities (106 beds and 127 beds, respectively), recorded high MAR indices (0.592 and 0.683). This may imply that the resistance rate largely depends on the regularly prescribed classes of antibiotics and the presence of different departments at each hospital as opposed to the number of patients accommodated in the facilities. All the isolates and all the sites reported multiple antibiotic resistance index values higher than 0.2. MAR index values greater than 0.2 indicate a high level of antibiotic contamination at the source (Osundiya et al. 2013). The elevated MAR index values observed in *E. coli, Klebsiella* species, *E. cloacae,* and *Citrobacter* species are consistent with the MAR index values reported in the same members of Enterobacterales isolated from urinary tract infections in a tertiary-care hospital in Hungary in a surveillance study conducted between 2008 and 2017, where *E. coli* and *Klebsiella* species reported higher MAR index values compared to CES (*Citrobacter*, *Enterobacter,* and *Serratia*) (Gajdács 2019). Notably, there was an enrichment of the ARB in the sewage sludge after thermophilic digestion (MAR index 0.560, from 0.500 in the activated sludge). The detection of increased antibiotic resistant bacteria in wastewater treatment plants' effluent has been reported in other studies, (Galler et al. 2014, Hocquet et al. 2016, White et al. 2016). However, the increase in resistance development among susceptible bacteria facilitated by WWTP processes has not yet been established (Galvin et al. 2010).

Available data suggest that β-lactam agents (especially penicillins and cephalosporins) are the most frequently used class of antibacterial agents across Europe in both hospital and community settings (Connor et al. 2017, Penalva et al. 2019). This is also supported by the annual antibiotic consumption data obtained from the PTE Central Clinical Pharmacy in the city of Pecs, Hungary, which showed high consumption of the β-lactam agents in the years 2006 and 2019 compared to the other antibiotic classes. This is suggestive of a rise in resistance development among these

agents. Although our study used ceftriaxone to screen for the β-lactam resistant enteric bacteria, high resistance rate to other third-generation cephalosporins observed was attributed to cross-resistance and not necessarily to the frequency of use. High levels of resistance to the same antimicrobial agents (cefpodoxime, cefotaxime, and ceftazidime) in Enterobacterales were found in effluents from WWTPs in Navarra, Northern Spain (Ojer-Usoz et al. 2014a). *bla*$_{\text{CTX-M}}$ type ESBL observed in *E. coli*, *K. pneumoniae* and *C. freundii* in this study was largely responsible for resistance to extended-spectrum cephalosporins as reported in previous studies (Coque et al. 2008a).

When comparing the plasmid sequences from 21 selected isolates, a cluster of *bla*$_{\text{CTX-M-27}}$ harboring *E. coli* and another group of *bla*$_{\text{CTX-M-15}}$ carrying *K. pneumoniae* isolates were revealed. In Hungary, CTX-M-15 and CTX-M-27 are found to be the dominant ESBL types among clinical isolates of *K. pneumoniae* and *E. coli*, respectively, which is in correspondence with our findings (Nagy et al. 2021, Tóth et al. 2022). Considering that the *bla*$_{\text{CTX-M-27}}$ harboring *E. coli* and *bla*$_{\text{CTX-M-15}}$ carrying *K. pneumoniae* isolates identified in the hospital and nursing home effluents can be of fecal origin from patients and nursing home residents, it can be presumed that their dominance in our samples resembles their prevalence among local inhabitants. The highly identical contigs shared within a cluster raises the possibility of clonal relatedness of the isolates. Unfortunately, this question could not be addressed, because the DNA samples subjected to next generation sequencing were enriched for plasmids, and therefore the coverage of chromosomal fragments was too low to be suitable for MLST analysis. Besides the two major clusters, CTX-M-15 producing *C. freundii*, CTX-M-1 producing *E. coli*, SHV-12 producing *K. pneumoniae*, and CTX-M-30 producing *K. oxytoca* were also detected in our study. The majority of the isolates carried multiple antibiotic resistance genes, and many of these genes occurred to be co-located on defined contigs. These findings might explain the high frequency of associated/co-resistance and elevated MAR indices revealed in this study.

The ESBL producers were observed more frequently in hospital effluents and WWTP, which appears to mirror similar observations made in South Africa, Tunisia, and Spain, reporting high rates of ESBL prevalence from hospital effluent and urban wastewater treatment plants (Sghaier et al. 2019, King et al. 2020). The presence of a high proportion of ESBL producers observed among isolates from hospital effluents may suggest increased prescription of certain extended-spectrum ß-lactam antimicrobials. Hsu et al. (2010) observed a significant increase in prescription

of certain extended-spectrum β-lactam antibiotics, which were associated with high levels of ESBL producers in hospital effluents in Singapore The *E. cloacae* species were non-ESBL producers and showed resistance to cefoxitin (a cephamycin), which can be supported by the observation that ESBL-producing *E. cloacae* are less prevalent and hence rarely reported as most *Enterobacter* species carry AmpC cephalosporinases, which are not inhibited by clavulanic acid (Ojer-Usoz et al. 2014b). However, other Enterobacterales recored remarkably low resistance to cefoxitin. This observation may be explained by the fact that cefoxitin does not have a marketing authorization in the country (Hungary), therefore it is not used in the clinics, implying non exposure to microbes hence the low resistance observed.

Although carbapenemases were not reported in the plasmid DNA of Enterobacterales isolates, a *bla*$_{\text{VIM}}$ gene was detected among the *Klebsiella oxytoca* isolates by colony PCR. This is in support of certain reports regarding the emergence of carbapenemase-producing *Klebsiella* spp. from environmental samples (Isozumi et al. 2012, Thomas et al. 2013). *Klebsiella* species harboring the *bla*$_{\text{VIM}}$ gene have been previously reported among isolates at the Clinical Centre University of Pécs (Melegh et al. 2014b), which is located within this same catchment area, suggesting that hospital effluents may be reservoirs of carbapenemase producers that can be linked to clinical sources. The high rate of susceptibility to meropenem observed in this study is consistent with a similar observation regarding low carbapenem resistance in Enterobacterales reported from wastewater treatment plants (Ojer-Usoz et al. 2014a). Clinical surveillance data in a tertiary care hospital in Hungary among Enterobacterales for the period 2004–2015 reported zero resistance to carbapenems; imipenem, meropenem, and ertapenem (Magyar et al. 2017). This low rate of carbapenems resistance can further be explained by the fact that their consumption in the year 2019 during which this study was conducted appeared to be extremely low based on the annual consumption data obtained from the PTE Central clinical Pharmacy where only 0.9% and 2.4% annual consumption rate was reported for meropenem and imipenem, respectively.

Fluoroquinolones hold the fifth position in the European antimicrobial market, with a maximum of 3.04 DDD (defined daily dose/1000 inhabitants) (Penalva et al. 2019). Our findings showed a high rate of resistance to ciprofloxacin, consistent with previously reported resistance to fluoroquinolones among isolates from various environmental compartments (Da Costa et al. 2006, Moore et al. 2008). Increased resistance to fluoroquinolones among Enterobacterales from urinary tract infections has been reported in clinical surveillance data (Magyar et al. 2017, Gajdács 2019).

Consistent with our finding, a recent study in South Africa also found an increased rate of co-resistance between third-generation cephalosporins and fluoroquinolones in *Klebsiella* spp. (King et al. 2020). Other studies have demonstrated remarkable co-resistance to fluoroquinolones and broad-spectrum cephalosporins among *E. coli* and *K. pneumoniae* isolated from wastewater (Conte et al. 2017). The presence of the quinolone resistance protein *qnrB1* and the aminoglycoside modifying enzyme *aac(6')-Ib-cr* variant associated with low-level fluoroquinolone resistance identified in *Klebsiella* isolates indicates that acquired genes contribute to fluoroquinolone resistance among *Klebsiella* spp. from the environment. Ciprofloxacin resistance among *E. coli* was, however, mainly attributed to accumulation of double serine mutations in the DNA gyrase and topoisomerase IV genes, as reported by Fuzi et al (2017) since they did not carry acquired resistance genes.

Similarly, resistance to gentamicin in this study occurred frequently among *Klebsiella* strains, which is consistent with a previous observation in *Klebsiella* spp. from wastewater treatment plants and hospital effluents in KwaZulu-Natal, South Africa (King et al. 2020). $bla_{\text{CTX-M}}$ harboring plasmids are often known to carry other genes of resistance, particularly to aminoglycosides, tetracycline, sulfonamides, and trimethoprim, suggesting co-selection, co-expression, and hence co-resistance (Pai et al. 2006). This finding can be linked to plasmid encoded aminoglycoside modifying enzyme encoding genes, *aph(3'')-Ib* and *aph(6)-1d* (phosphotransferases), *aadA1* and *aadA5* (adenylyltransferases), and *aac(3)-IIa* (acetyltransferase), which were identified in *E. coli* and *Klebsiella* spp, some of which are associated with gentamicin and tobramycin resistance. An increased rate of resistance to sulfamethoxazole/trimethoprim among the isolates can be associated with sulfonamide resistance genes (*sul1* and *sul2*) and *dfrA* (*dfrA14* and *dfrA17*) expressing dihydropteroate synthase and dihydrofolate reductases responsible for target replacement, conferring resistance to sulfonamides and diaminopyrimidines. A recent clinical surveillance data on *E. coli* from urinary tract infections indicated a high resistance rate of sulfamethoxazole/trimethoprim (Gajdács 2019), implying that the resistance observed in wastewater isolates may be clinical in origin.

It is notable that even though the isolates originated from different spots of the wastewater system, they were found to carry more or less the same plasmid groups. The main plasmids were the IncF replicons and their subtypes (FIA, FIB, and FII) which were evident in all the sequenced isolates. Acquired antibiotic resistance genes in bacteria are frequently carried on plasmids, with F plasmids

being the most common conjugal plasmids in Enterobacterales linked to antibiotic resistance ("R factors") (Moran et al. 2015). According to Stephens et al (2020), antibiotic resistance genes have been found in plasmids with a narrow host range, including IncI complex replicons (Z, B/O, K, or I1), but the majority of antibiotic resistance genes were associated with F replicons, and in most cases, multiple subtypes of F replicons were found on the same plasmids. F- and I-complex replicons are frequently found in association with conjugating plasmids (Stephens et al. 2020). ESBL genes, carbapenemase genes, genes coding aminoglycoside-modifying enzymes, and plasmid-mediated quinolone resistance (PMQR) genes are the most frequently described resistance genes on IncF plasmids (Rozwandowicz et al. 2018).

Multiple studies have described multidrug resistance in Enterobacterales, which is in line with the high frequency of strains exhibiting multiple antibiotic resistance phenotype among our isolates. Resistance to three or four antimicrobial classes was observed, with the majority of the isolates recording resistance to three classes, while a few isolates mainly *E. coli,* showed resistance to the four antibiotic families tested. Our findings reflect those reported by Rabbani et al (2017) where over 60% of *E. coli* isolates from untreated hospital wastewater were multidrug resistant. Estrada-Garcia et al (2005) reported multidrug resistance in approximately 58% of *E. coli.* Similarly, in a study involving 40 strains of *E. coli* isolated from the liquid hospital waste at Chittagong Medical College Hospital in Bangladesh, all were found to be multi-drug resistant (≥4) (Islam et al. 2008). Multiple antibiotic resistant *K. pneumoniae* from wastewater have also been reported in a recent study in KwaZulu-Natal, South Africa (King et al. 2020). In the clinical environment similar to wastewater, surveillance data have reported a significant increase in MDR (≥4 antibiotic classes) among uropathogens, including *E. coli*, *K. pneumoniae,* and *P. mirabilis* [46]. Multiresistance has been associated with the co-occurrence of resistance genes on mobile genetic elements where traits for resistance to multiple antimicrobials occur in particular plasmids and the same mechanism happens to be active against a wide spectrum of antimicrobials (Rabbani et al. 2017).

Multiple antibiotic resistance phenotype occurred at high frequency in the hospitals and the nursing home where individuals are likely to be put on a treatment regimen on a regular basis. In a related study, the percentage of multiple drug resistance in *E. coli* was higher in a nursing home than in hospital effluents (Oberlé et al. 2012). Co-resistance between cephalosporin and ciprofloxacin in this study was more frequent among isolates from the four hospitals, while that of cephalosporin and trimethoprim/sulfamethoxazole occurred more frequently among those from the nursing

home. These findings may be related to antimicrobial drug prescriptions and demonstrate that antimicrobial drug resistant bacteria are likely to be selected in the human gastro-intestinal tract due to antimicrobial usage (Tenaillon et al. 2010). The discharge of untreated hospital effluent into the urban wastewater network for co-treatment with the rest of municipal wastewater at the WWTP before releasing it into the environment is a general practice across many countries in Europe (Morris et al. 2016). This may be directly linked to the high resistance rate observed in the hospital effluents. Isolates of clinical origin may disseminate resistance to environmental microbes, although resistant isolates from hospital effluents have not been correlated with those of clinical origin (White et al. 2016).

P. aeruginosa is a common opportunistic pathogen associated with significant morbidity and mortality, especially in cases of infections caused by MDR/XDR isolates in hospital settings (Potron et al. 2015). A few categories of antimicrobial agents are effective against *P. aeruginosa* infections due to its intrinsic, adaptive, and acquired antimicrobial resistance traits. They include aminoglycosides (gentamicin, tobramycin, amikacin, netilmicin), carbapenems (imipenem, meropenem), cephalosporins (ceftazidime, cefepime), fluoroquinolones (ciprofloxacin, levofloxacin), penicillin with β-lactamase inhibitors (BLI) (ticarcillin and piperacillin in combination with clavulanic acid or tazobactam), monobactams (aztreonam), fosfomycin and polymyxins (colistin, polymyxin B) (Barnes et al. 2018, Bassetti et al. 2018, Nguyen et al. 2018). This study tested *Pseudomonas* species against five of these agents (gentamicin, ciprofloxacin, ceftazidime, imipenem, and meropenem) where the isolates demonstrated increased resistance (>75%) to all except meropenem. This species is one of the clinically significant bacteria that has been shown to be resistant to a variety of antimicrobial classes including β-lactams, carbapenems, fluoroquinolones, aminoglycosides, and polymyxins (Johansen et al. 2008, Bergen et al. 2010). MAR was evident in all the samples, with a higher proportion seen in hospital effluents and wastewater treatment plant samples (MAR index ≤ 0.7). All the isolates from the various sources had MAR indices greater than 0.2, and MAR indices greater than this threshold indicate high-risk source of antibiotic contamination (Osundiya et al. 2013), suggesting that frequent use of antibiotics in hospitals and the community may have contributed to this observation. Multiple antimicrobial resistance in this bacterium from various sources is widespread and it is known to exhibit acquired resistance mechanisms involving genetically imported resistance determinants via horizontal gene transfer (Kraemer et al. 2019). Various intrinsic multiple resistance mechanisms

to antibiotics, including decreased permeability of the outer membrane, overexpression of efflux systems, modified penicillin-binding proteins, chromosomal β-lactamase production of antibiotic inactivating enzymes and target modifications, all contribute to resistance to multiple antibiotics, including β-lactams, aminoglycosides, polymyxins and fluoroquinolones (Oie et al. 2009, Santajit and Indrawattana 2016, Memar et al. 2019). The increased resistance to gentamicin and ciprofloxacin observed in this study was contrary to an earlier finding where very high sensitivity of *Pseudomonas* species from WWTP to gentamicin and ofloxacin (a fluoroquinolone) was reported (Odjadjare et al. 2012), suggesting that aminoglycosides and fluoroquinolones have become less efficacious against *P. aeruginosa* strains due to growing resistance. Our findings mirror those reported in studies where high resistance to gentamicin and ciprofloxacin in *Pseudomonas* strains isolated from pharmaceutical effluents and the clinical environment was observed (Lateef 2004, Navon-Venezia et al. 2005). On the contrary, a clinical surveillance study on uropathogens including *P. aeruginosa* in a tertiary care hospital in Hungary between 2004 and 2015 showed a significant decrease in gentamicin and ciprofloxacin resistance rates (Magyar et al. 2017). *Pseudomonas* resistance to broad-spectrum cephalosporins (cefotaxime and cefepime) was reported in a previous study (Odjadjare et al. 2012), and resistance to these broad-spectrum cephalosporins is commonly associated with production of molecular Class A ESBLs of TEM, SHV and CTX-M types, which are not common in *P. aeruginosa (*Picão et al. 2009*).* Although 25 isolates expressed β-lactamase hydrolysis phenotypically, only two isolates carrying the TEM and CTX-M types were identified. These ESBL Enterobacterales types of enzymes have been described in *P. aeruginosa*, and are mostly linked to genetic importation following lateral gene transfer (Chanawong et al. 2001). *Pseudomonas* species are members of the "SPACE" (*Serratia* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Citrobacter* spp., *Enterobacter* spp.) organisms, characterized by inducible AmpC-based resistance (MacDougall 2011, Narayanan et al. 2016). It is suggestive that resistance to ceftazidime among the majority of the isolates was attributed to this mechanism and possibly to broad-spectrum carbapenem degrading enzymes. Carbapenem resistance (imipenem and meropenem), which was of major interest in this study, was found in more than 58% of the isolates, despite the fact that these antibiotics are often regarded safe therapeutic options of the last resort in *P. aeruginosa* infections due to their high affinity for penicillin-binding proteins, stability against extended-spectrum β-lactamases, and permeability of bacterial outer membranes (Zavascki et al. 2010). Carbapenem resistance has been observed to be

on an upward trend in non-fermenting Gram-negative bacteria, including *Pseudomonas* spp. which is associated with possible resistance mechanisms including degrading enzymes, modifications in the transpeptidases, porin deletion and the overexpression of efflux transporters (Zeng et al. 2014, Ruppé et al. 2015, Bassetti et al. 2018). Carbapenem resistance in *P. aeruginosa* isolates in this study was largely associated with the metallo-β-lactamase VIM-4, which was detected in the carbapenem resistant isolates. One of these environmental isolates carried both VIM-4 and NDM encoding genes. The occurrence of two enzymes (KPC and NDM-1) in clinical isolates of *P. aeruginosa* linked to hospital acquired infections has also been reported (Paul et al. 2015). According to published data, the prevalence of metallo-β-lactamase enzymes in *P. aeruginosa* isolates resistant to carbapenems ranges from 2%–100% (Rodríguez-Martínez et al. 2009). These enzymes form the dominant class of carbapenemases found in *P. aeruginosa*, with the most common ones being; VIM, IMP, NDM, GIM, and SPM, where in most cases, the enzymes are encoded on highly mobile genetic elements including plasmids, integrons, and gene cassettes which mediate their transmission (Walsh et al. 2005). Although our study found VIM-4 as the predominant gene in *P. aeruginosa* from all the carbapenem resistant wastewater samples, a previous study on *P. aeruginosa* found isolates harboring mainly the enzyme IMP (Slekovec et al. 2012). In earlier reports, isolates of *P. aeruginosa* from WWPT and *Klebsiella* spp. carrying the VIM-4 variant were recovered from the Clinical Centre University of Pécs (Libisch et al. 2006, Melegh et al. 2014b) which formed part of our wastewater sampling spots. The occurrence of VIM-4 positive isolates of *P. aeruginosa* from the wastewater samples may be traced to clinical sources since molecular studies have confirmed the relationship between clinical and environmental isolates with the growing evidence that environmental sources have likely contributed to the acquisition of antibiotic resistance genes found in human microbial communities (Wright 2010, Rozman et al. 2020).

7.0 CONCLUSIONS

We established the presence of antimicrobial pharmaceuticals namely, macrolides (azithromycin, clarithromycin), fluoroquinolones (ciprofloxacin, norfloxacin and ofloxacin), sulfonamides (sulfadiazine, sulfamethoxazole) and trimethoprim at significantly varied concentrations in wastewater from the various sources, with trimethoprim and fluoroquinolones being the dominant compounds, mainly from the hospital drainage systems.

We determined a 2 fold higher cfu count of the Gram-negative bacteria resistant to broad-spectrum cephalosporin (ceftriaxone) in hospital and nursing home effluents compared to that of wastewater treatment plant and the urban wastewater, while the cfu count of carbapenem-(imipenem) resistant Gram-negative bacteria was 8 fold higher in the hospital and nursing home effluents than that of the wastewater treatment plant and the urban wastewater. However, in comparison, resistance to ceftriaxone was higher than that of imipenem in samples across all the locations.

We established a widespread multidrug resistance among enteric bacteria and *P. aeruginosa* in wastewater, especially from the hospital and sanitary sources and the resistance rate was enriched at the biological reactor and further during mesophilic digestion of the sewage sludge.

We established a positive correlation between the concentration of fluoroquinolone residues (ciprofloxacin and ofloxacin) in wastewater and resistance to ciprofloxacin in *P. aeruginosa* isolates which points to possible selection of resistance created by antimicrobials in wastewater systems.

We established a similar resistance rate of β-lactam antibiotics among Enterobacterales from environmental sources (although these drugs are considered the backbone of antibiotic therapy making them the most widely used antibiotics in clinical practice) as other classes of antimicrobials, namely fluoroquinolones, aminoglycosides and sulfonamides, which can be linked to simultaneous transmission of plasmid encoded genes. However, carbapenems (imipenem and meropenem) remain the most potent antimicrobials against enteric bacteria with carbapenem resistance in *P. aeruginosa* observed to be on the upward trend.

We determined the presence of multiresistant members of Enterobacterales harboring plasmid-mediated extended-spectrum β-lactamases mainly of CTX-M and SHV types (that degrade broad-spectrum cephalosporins) in wastewater. The broad-spectrum β-lactamase producers occurred at a higher frequency in hospital effluents than in wastewater from other sources. Their presence can be attributed either to the development of resistance in the source population, and/or its build-up in the environment through selection pressure as well as resistance dissemination of the phenotype via horizontal gene transfer.

We established that *E. coli* isolates from wastewater harbor ESBL of mainly $bla_{\text{CTX-M-27}}$ whereas *Klebsiella* spp, harbor mainly $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-30}}$, and $bla_{\text{SHV-12}}$ variants. Similarly, Metallo-β-lactamase VIM-4 was the main enzyme responsible for the inactivation of the carbapenems in *P. aeruginosa* from the wastewater. The reporting of bla_{VIM-4} in isolates of *P. aeruginosa* from wastewater and the same gene variant previously reported from clinical isolates from one of the healthcare facilities in the city points to a direct connection between resistance in clinical isolates and their environmental counterparts. On the other hand, detection of bla_{NDM} in one of the *P. aeruginosa* isolates indicates the possibility of the spread of this gene in the environment.

We observe that continuous exposure of the environment to antimicrobial residues may lead to the emergence of resistant strains, and soil amendment with sewage sludge carrying resistant determinants may result in the buildup of environmental reservoirs of ESBL and carbapenemase producing bacteria emanating from the pool of resistance genes circulating in the broad microbial population entering the ecosystem.

Overall, this study demonstrates the presence of antimicrobial pharmaceuticals in wastewater and abundance of multidrug resistant enteric bacteria and *P. aeruginosa* in the same environment. This wastewater has direct connection to human sources, especially the clinical wastewater where the resistance prevalence is higher. The hospital effluents discharged into municipal wastewater network without pretreatment present an ideal vehicle for the carriage of bacteria of both human and environmental origin harboring antibiotic resistance genes. A proportion of these antibiotic-resistant Gram-negative bacteria is enriched at the biological reactor as wastewater treatment progresses. This potentiates a public health threat posed by the disposal of non-decontaminated digested sewage sludge into the environment for soil amendment because it may expose antibiotic resistant bacteria and their resistance genes to human and animal hosts. Therefore, it is desirable

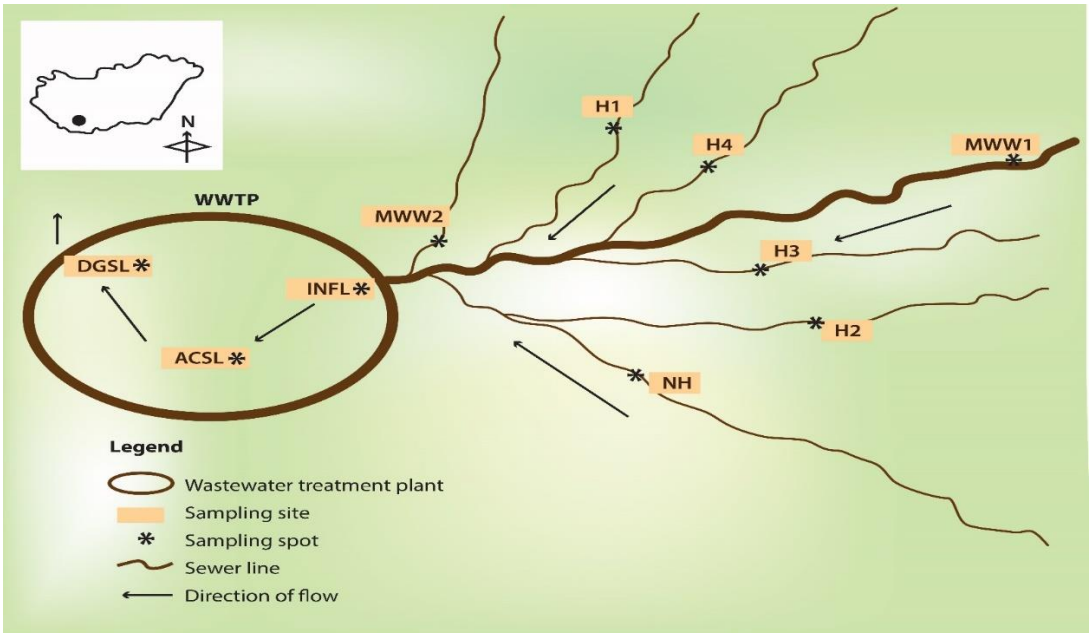
to disinfect such hospital wastewater and subsequently decontaminate the digested sewage sludge prior to disposal. These would be feasible management strategies to minimize the risk of environmental contamination with antibiotic resistant bacteria and to slow the progression of antimicrobial resistance in the environment. The findings of this study demonstrate that monitoring wastewater of anthropogenic origin presents a promising strategy for generating valuable data which can be correlated to the prevalence of clinically important resistant bacteria from the source population and may provide a cheaper alternative in regions (especially low-income countries) facing challenges that limit clinical surveillance.

Összefoglalás

Az antibiotikumok nagy mennyiségű használata miatt az olyan „reaktorok”-ban, mint a humán bióta, a kórházak, szennyvizek és szennyíztisztító telepek, a talaj- és szennyvíziszapok, az antibiotikumjelenlét által okozott szelekciós nyomás következtében kiszelektálódnak az antibiotikumokkal szemben rezisztens mikrobák. Ezen antibiotikum nyomás hatására a mikroorganizmusok már felszerelkeztek, valamint folyamatosan felhalmoznak az antibiotikumok hatásának ellenálló (rezisztencia) géneket (ARG). Ezen velünk élő mikrobák közé tartozik a *Staphylococcus* nemzetség számos faja, amelyek a humán bőrfelszínen megtalálhatók, vagy az *Enterobacteriaceae* család tagjai, amelyek a humán bélflóra mikrobiótájának részei, mint az *E. coli*, *Klebsiella pneumoniae* és *Enterobacter* törzsek. A környezetünkben, talajban, vizes környezetben és helyiségekben általánosak az *Acinetobacter baumanni*, *Pseudomonas aeruginosa* valamint a *Stenotrophomonas maltophilia* baktériumok, ezek törzsei is egyre nagyobb számban okoznak az egészségügyi ellátással összefüggő (nozokomiális) fertőzést Magyarországon. Vizsgálatainkat 2018-ban kezdtük el, amely során kórházi szennyvizek, egy ápolási otthon szennyvizének, valamint a szennyvíztelep befolyó szennyvizének, az oxigénes reaktor vizének, valamint a biogáztartály fermentorából távozó erjesztett szennyvíziszapból vett mintákban vizsgáltuk különböző osztályokba tartozó antibiotikumokkal szemben mutatott rezisztenciáját *Escherichia*, *Klebsiella*, *Enterobacter* és *Pseudomonas* törzseknek.

A fő cél az *Enterobacterales* és *Pseudomonas aeruginosa* rendbe tartozó széles spektrumú β-laktamáz (ESBL) és karbapenenamáz termelő Gram-negatív opportunista kórokozók izolálása és jellemzése volt kórházi szennyvízből, települési szennyvízből és szennyvíztisztító telepről (befolyó, oxigénes reaktor, erjesztett iszap), valamint ugyanazon szennyvízmintákban az antibiotikumvegyületek jelenlétének és koncentrációjának meghatározása.

A szennyvízmintákban meghatároztuk az antimikrobiális hatóanyagok, makrolidok (azitromicin, klaritromicin), fluorokinolonok (ciprofloxacin, norfloxacin és ofloxacin), szulfonamidok (szulfadiazin, szulfametoxazol) és trimetoprim, jelentősen eltérő mennyiségét tapasztaltuk az antibiotikumoknak a különböző forrásokból. A mintákban a trimetoprim és fluorokinolonok voltak a domináns vegyületek, főként a kórházi szennyvizekben. A következő ábra a mintavételi helyeket mutatja be Pécs városán belül.



A mintavételi helyeket ábrázoló sematikus diagram. H1–H4, kórházi szennyvíz; NH, idősek otthona; MWW1 és MWW2, csak lakossági szennyvíz; INFL, befolyó szennyvíz; ACSL, aerob reaktor, eleveniszap; DGSL, rothasztott iszap; WWTP: Szennyvíztisztító telep.

A széles spektrumú cefalosporinokkal (ceftriaxon) szemben rezisztens Gram-negatív baktériumok 2-szer magasabb élősejtszámát (cfu) mutattuk ki a kórházi és az ápolási otthon szennyvízében, összehasonlítva a szennyvíztisztító telep és a települési szennyvíz mintáival, míg a karbapenem (imipenem) rezisztens Gram-negatív cfu érték 8-szor magasabb volt szintén a kórházi és idősotthoni szennyvízben, mint a szennyvíztisztító telep és a települési szennyvíz mintáiban.

Összehasonlításképpen azonban a ceftriaxonnal szemben rezisztens mikrobák száma minden mintavételi helyen magasabb volt, mint az imipenemmel szembeni kimutatott rezisztens mikrobák száma.

Megállapítottuk, hogy széles körben elterjedt a multidrog-rezisztencia az enterális baktériumok valamint a *P. aeruginosa* baktériumok körében, különösképpen a kórházi és az egészségügyi intézmények szennyvízmintáiban, a rezisztens mikrobák aránya az aerob reaktorban és a szennyvíziszap mezofil erjesztése során tovább dúsult.

Pozitív korrelációt állapítottunk meg a szennyvízben található fluorokinolon maradványok (ciprofloxacin és ofloxacin) koncentrációja és a *P. aeruginosa* izolátumok ciprofloxacin

rezisztenciája között, ami a szennyvízrendszerekben az antimikrobiális szerek által létrehozott rezisztencia lehetséges szelekciójára utal.

Hasonló rezisztenciaarányt állapítottunk meg a β-laktám antibiotikumok esetében a szennyvízmintákból izolált Enterobacterales törzsek között (bár ezeket a gyógyszereket tekintik az antibiotikumterápia gerincének, így a klinikai gyakorlatban a legszélesebb körben alkalmazott antibiotikumok), mint az antimikrobiális szerek más osztályainál, nevezetesen a fluorokinolonok, aminoglikozidok és szulfonamidok esetében, amelyek a plazmid által kódolt gének szimultán átviteléhez köthetők.

A saját és más kutatók eredményei alapján mondhatjuk, hogy a karbapenemek (imipenem és meropenem) továbbra is a legerősebb antimikrobiális szerek az enterális baktériumok ellen, mivel szinte „extrém" mértékben érzékenyek velük szemben. Ezzel szemben viszont a *P. aeruginosa* törzsek nagyobb számban mutattak rezisztenciát ezen antibiotikumokkal szemben, sőt a karbapenem rezisztenciával rendelkező *P. aeruginosa* törzsek száma növekvő tendenciát mutat. Ez azért sajnálatos, mivel ezen antibiotikumokat főleg akkor használják, ha más hatóanyagokkal kudarcot vallottak a gyógyítás során.

Meghatároztuk az Enterobacterales multirezisztens törzsekben a plazmidközvetített széles spektrumú β-laktamázokat, elsősorban CTX-M és SHV típusba tartozó törzseinek jelenlétét a szennyvízben. A széles-spektrumú β-laktamáz termelők nagyobb gyakorisággal fordultak elő a kórházi szennyvízben, mint az egyéb forrásokból származó mintákban. A jelenlétük tulajdonítható egyrészt annak, hogy a rezisztencia már kialakult azokban az egyedekben, amelyek a forráspopulációt alkotják, mint a humán mikrobiom, vagy a szennyvíz. Kialakulhatott a rezisztencia a környezetben, ahol az antibiotikumok jelenléte a szubinhibitori koncentráció miatt szelekciós nyomást váltott ki, és ezek a mutánsok elszaporodtak. Ez általában gyakori jelenség az antimikrobiális szerekkel terhelt környezetben. Ebben az esetben az ESBL géneket hordozó enterobaktériumok felhalmozódását jelenti különböző okok miatt, mint pl. a szelekciós nyomás és/vagy a horizontális géntranszfer mechanizmusa miatt. Az ESBL géneket hordozó enterobaktériumok nagy számának jelenléte a genetikai cseremechanizmusok, különösen a konjugáció révén következhettek be, ahol az általunk izolált baktériumok horizontális géntranszfer révén importálhatták ezeket a géneket más baktériumoktól ugyanabban a környezetben, ebben az esetben a szennyvízben.

Megállapítottuk, hogy a szennyvízből izolált *E. coli* törzsek az ESBL gének közül főleg a $bla_{\text{CTX-M-27}}$-t, míg a *Klebsiella* törzsek főleg a $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-30}}$ és a $bla_{\text{SHV-12}}$ variánsokat hordozták. Hasonlóképpen a metallo-β-laktamáz VIM-4 volt a fő enzim, amely felelős a *P. aeruginosa* törzsekben a karbapemenek inaktíválásáért. A *P. aeruginosa* szennyvízből származó izolátumaiban ugyanazt a $bla_{\text{VIM-4}}$ génváltozatot mutattuk ki, mint amelyet a Pécsi Egyetem Klinikai Központjában egy klinikai izolátumban mutattak ki Melegh és munkatársai (Melegh et al. 2014a, 2014b). A VIM-4 variánst először Libisch és munkatársai (2006) azonosították egy klinikai izolátumban. VIM-4 variáns leírása ebben a két Gram-negatív baktériumban, mind a környezeti mintából (szennyvíz), mind a klinikai forásból Libisch és Melgh által (Libisch et al 2006, Melegh et al. 2014b) azt mutatja, hogy közvetlen kapcsolat van a klinikai környezetben kialakult rezisztencia és a környezetben, a klinikai egységen kívül talált rezisztencia között. Mivel a molekuláris vizsgálatok megerősítették a kapcsolatot a klinikai és a környezeti izolátumok között, valamint a környezeti rezisztencia klinikai forrásokra vezethető vissza. Másrészt a bla_{NDM} kimutatása a *P. aeruginosa* izolátumok egyikében azt jelzi, hogy a gén elterjedhet a környezetben.

Megfigyeltük, hogy a környezet folyamatos antimikrobiális szermaradványokkal történő terhelése rezisztens törzsek kialakulásához vezethet, a talaj feljavítása rezisztens törzseket hordozó szennyvíziszappal pedig ESBL és karbapenemázt termelő baktériumok környezeti tárolóinak (Reservoir) felhalmozódását eredményezheti, amely az ökoszisztémába kerülő széles mikrobapopulációban keringő (cirkuláló) rezisztenciagének készletéből ered.

Összeségében ez a tanulmány bemutatja és igazolja az antimikrobiális hatóanyagok és a multidrogrezisztens enterális baktériumok, valamint *P. aeruginosa* törzsek bőségének jelenlétét a szennyvízben, ugyanabban a környezetben. Ez a szennyvíz közvetlen kapcsolatban van az emberi forrássokkal, különösen a klinikai szennyvízzel, ahol a rezisztencia előfordulásának gyakorisága magasabb. A kórházak kifolyó szennyvízének előkezelés nélkül történő kibocsátása a lakossági szennyvízhálózatba ideális eszközt jelent az antibiotikumrezisztens géneket hordozó emberi és környezeti eredetű baktériumok szállítására. Ezen antibiotikumrezisztens Gram-negatív baktériumok egy része feldúsul a biológiai reaktorban a szennyvíztisztítás folyamata során. Ez a tény felerősíti azt a közegészségügyi fenyegetést, amelyet a nem fertőtlenített erjesztett szennyvíziszap talajjavítás céljából a környezetbe történő elhelyezése jelent, akkor az lesz az antibiotikum rezisztens baktériumok és azok rezisztencia génjeinek forrása, kitéve az emberi és

állati gazdaszervezeteket ezen mechanizmusoknak. Ezért kívánatos az ilyen kórházi szennyvizek fertőtlenítése, majd az emésztett szennyvíziszap fertőtlenítése az ártalmatlanítás előtt. Ezek megvalósítható kezelési stratégiák lennének az antibiotikum-rezisztens baktériumokkal való környezetszennyezés kockázatának minimalizálására és az antimikrobiális rezisztencia előrehaladásának lelassítására a környezetben. A tanulmány eredményei azt mutatják, hogy az antropogén eredetű szennyvizek monitorozása ígéretes stratégia értékes adatok előállítására, amelyek összefüggésbe hozhatók a klinikailag fontos rezisztens baktériumok előfordulási gyakoriságával a forráspopulációból, és olcsóbb alternatívát jelenthet a régiókban. Különösen az alacsony jövedelmű országokban olyan kihívásokkal kell szembenézni, amelyek korlátozzák a klinikai felügyeletet.

(Fordította: Dr. Gazdag Zoltán)

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9.0 REFERENCES

Aali, R., M. Nikaeen, H. Khanahmad, and A. Hassanzadeh. 2014. Monitoring and comparison of antibiotic resistant bacteria and their resistance genes in municipal and hospital wastewaters. *International Journal of Preventive Medicine* **5**:887.

Al Aukidy, M., P. Verlicchi, A. Jelic, M. Petrovic, and D. Barcelò. 2012. Monitoring release of pharmaceutical compounds: occurrence and environmental risk assessment of two WWTP effluents and their receiving bodies in the Po Valley, Italy. *Science of the Total Environment* **438**:15-25.

Aldred, K. J., R. J. Kerns, and N. Osheroff. 2014. Mechanism of quinolone action and resistance. *Biochemistry* **53**:1565-1574.

Alexandrino, M., E. Grohmann, R. Szewzyk, and U. Szewzyk. 2007. Application of culture-independent methods to assess the bacteria removal efficiency of subsurface flow constructed wetlands. *Water Science Technology* **56**:217-222.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**:403-410.

Ambler, R. P. 1980. The structure of beta-lactamases. *Biological Science* **289**:321-331.

Andrews, J. M., and B. W. P. o. S. Testing. 2009. BSAC standardized disc susceptibility testing method (version 8). *Journal of Antimicrobial Chemotherapy* **64**:454-489.

Andrews, S., F. Krueger, A. Segonds-Pichon, L. Biggins, C. Krueger, and S. Wingett. 2020. FastQC: a quality control tool for high throughput sequence data [Online]. 2010. Available from: http://www. bioinformatics. babraham. ac. uk/projects/fastqc/. Accessed 2022 **27**.

Arango-Argoty, G., E. Garner, A. Pruden, L. S. Heath, P. Vikesland, and L. Zhang. 2018. DeepARG: a deep learning approach for predicting antibiotic resistance genes from metagenomic data. *Microbiome* **6**:23.

Azanu, D., B. Styrishave, G. Darko, J. J. Weisser, and R. C. Abaidoo. 2018a. Occurrence and risk assessment of antibiotics in water and lettuce in Ghana. *Science of the Total Environment* **622**:293-305.

Azanu, D., B. Styrishave, G. Darko, J. J. Weisser, and R. C. Abaidoo. 2018b. Occurrence and risk assessment of antibiotics in water and lettuce in Ghana. *Science of the Total Environment* **622-623**:293-305.

Baquero, F., J. L. Martinez, and R. Canton. 2008. Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology* **19**:260-265.

Barnes, M. D., M. A. Taracila, J. D. Rutter, C. R. Bethel, I. Galdadas, A. M. Hujer, E. Caselli, F. Prati, J. P. Dekker, K. M. Papp-Wallace, S. Haider, and R. A. Bonomo. 2018. Deciphering the Evolution of Cephalosporin Resistance to Ceftolozane-Tazobactam in Pseudomonas aeruginosa. *Molecular Biology* 9(6): e02085-18

Bartlett, J. G. 2005. 2005-6 Pocket book of infectious disease therapy. Lippincott Williams & Wilkins.

Bartrons, M., and J. Penuelas. 2017. Pharmaceuticals and Personal-Care Products in Plants. *Trends in Plant Science* **22**:194-203.

Bassetti, M., A. Vena, A. Croxatto, E. Righi, and B. Guery. 2018. How to manage *Pseudomonas aeruginosa infections*. *Drugs Context* **29**;7:212527

Batah, R., L. Loucif, A. O. Olaitan, N. Boutefnouchet, H. Allag, and J. M. Rolain. 2015. Outbreak of Serratia marcescens Coproducing ArmA and CTX-M-15 Mediated High Levels of

Resistance to Aminoglycoside and Extended-Spectrum Beta-Lactamases, Algeria. *Microbial Drug Resistance* **21**:470-476.

Bengtsson-Palme, J., D. G. J. Larsson, and E. Kristiansson. 2017. Using metagenomics to investigate human and environmental resistomes. *Journal of Antimicrobial Chemotherapy* **72**:2690-2703.

Bengtsson, P., E. Kristiansson, and D. Larsson. 2018. Environmental factors influencing the development and spread of antibiotic resistance. *FEMS Microbiology Reviews* **42**:1-41.

Bennett, P. M. 2008. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology* **153** Suppl **1**:S347-357.

Berendonk, T. U., C. M. Manaia, C. Merlin, D. Fatta-Kassinos, E. Cytryn, F. Walsh, H. Burgmann, H. Sorum, M. Norstrom, M. N. Pons, N. Kreuzinger, P. Huovinen, S. Stefani, T. Schwartz, V. Kisand, F. Baquero, and J. L. Martinez. 2015. Tackling antibiotic resistance: The environmental framework. *Nature Reviews Microbiology* **13**:310-317.

Bergen, P. J., J. B. Bulitta, A. Forrest, B. T. Tsuji, J. Li, and R. L. Nation. 2010. Pharmacokinetic/pharmacodynamic investigation of colistin against *Pseudomonas aeruginosa* using an in vitro model. *Antimicrobial Agents and Chemotherapy* **54**:3783-3789.

Berglund, B. 2015. Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics. *Infection Ecology and Epidemiology* **5**:28564.

Berglund, B., J. Fick, and P. E. Lindgren. 2015. Urban wastewater effluent increases antibiotic resistance gene concentrations in a receiving northern European river. *Environmental Toxicology Chemistry* **34**:192-196.

Birošová, L., T. Mackuľak, I. Bodík, J. Ryba, J. Škubák, and R. Grabic. 2014. Pilot study of seasonal occurrence and distribution of antibiotics and drug resistant bacteria in wastewater treatment plants in Slovakia. *Science of the Total Environment* **490**:440-444.

Blondiaux, N., O. Gaillot, and R. Courcol. 2010. MALDI-TOF mass spectrometry to identify clinical bacterial isolates: evaluation in a teaching hospital. *Pathologie Biologie* **58**:55-57.

Bolger, A. M., M. Lohse, and B. Usadel. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**:2114-2120.

Bonnet, R. 2004. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. *Antimicrobial Agents and Chemotherapy* **48**:1-14.

Bortolaia, V., R. S. Kaas, E. Ruppe, M. C. Roberts, S. Schwarz, V. Cattoir, A. Philippon, R. L. Allesoe, A. R. Rebelo, A. F. Florensa, L. Fagelhauer, T. Chakraborty, B. Neumann, G. Werner, J. K. Bender, K. Stingl, M. Nguyen, J. Coppens, B. B. Xavier, S. Malhotra-Kumar, H. Westh, M. Pinholt, M. F. Anjum, N. A. Duggett, I. Kempf, S. Nykasenoja, S. Olkkola, K. Wieczorek, A. Amaro, L. Clemente, J. Mossong, S. Losch, C. Ragimbeau, O. Lund, and F. M. Aarestrup. 2020. ResFinder 4.0 for predictions of phenotypes from genotypes. *Journal of Antimicrobial Chemotherapy* **75**:3491-3500.

Bradford, P. A. 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews* **14**:933-951.

Burrows, L. L. 2018. The Therapeutic Pipeline for *Pseudomonas aeruginosa* Infections. *ACS Infectious Diseases* **4**:1041-1047.

Bush, K., and G. A. Jacoby. 2010a. Updated functional classification of beta-lactamases. *Antimicrobial Agents and Chemotherapy* **54**:969-976.

Bush, K., and G. A. Jacoby. 2010b. Updated functional classification of β-lactamases. *Antimicrobial Agents and Chemotherapy* **54**:969-976.

Cao, J., Y. Hu, F. Liu, Y. Wang, Y. Bi, N. Lv, J. Li, B. Zhu, and G. F. Gao. 2020. Metagenomic analysis reveals the microbiome and resistome in migratory birds. *Microbiome* **8**:26.

Carattoli, A., E. Zankari, A. García-Fernández, M. Voldby Larsen, O. Lund, L. Villa, F. Møller Aarestrup, and H. Hasman. 2014. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrobial Agents and Chemotherapy* **58**:3895-3903.

Carraro, E., S. Bonetta, C. Bertino, E. Lorenzi, S. Bonetta, and G. Gilli. 2016. Hospital effluents management: Chemical, physical, microbiological risks and legislation in different countries. *Journal of Environmental Management* **168**:185-199.

Carvalho, I. T., and L. Santos. 2016a. Antibiotics in the aquatic environments: A review of the European scenario. *Environment International* **94**:736-757.

Carvalho, I. T., and L. J. E. i. Santos. 2016b. Antibiotics in the aquatic environments: a review of the European scenario. *Environment International* **94**:736-757.

Cassini, A., L. D. Hogberg, D. Plachouras, A. Quattrocchi, A. Hoxha, G. S. Simonsen, M. Colomb-Cotinat, M. E. Kretzschmar, B. Devleesschauwer, M. Cecchini, D. A. Ouakrim, T. C. Oliveira, M. J. Struelens, C. Suetens, D. L. Monnet, and A. M. R. C. G. Burden of. 2019. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *The Lancet Infectious Disease* **19**:56-66.

Chanawong, A., F. H. M'Zali, J. Heritage, A. Lulitanond, and P. M. Hawkey. 2001. SHV-12, SHV-5, SHV-2a and VEB-1 extended-spectrum beta-lactamases in Gram-negative bacteria isolated in a University hospital in Thailand. *Journal of Antimicrobial Chemotherapy* **48**:839-852.

Chartier, Y. 2014. Safe management of wastes from health-care activities. World Health Organization.

Cheesbrough, M. 1990. Medical laboratory manual for tropical countries. M. Cheesbrough, 14 Bevills Close, Doddington, Cambridgeshire, PE15 OTT.

Chen, M., K. Ohman, C. Metcalfe, M. G. Ikonomou, P. L. Amatya, and J. Wilson. 2006. Pharmaceuticals and endocrine disruptors in wastewater treatment effluents and in the water supply system of Calgary, Alberta, Canada. *Water Quality Research* **41**:351-364.

Chopra, I., and M. Roberts. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews* **65**:232-260.

Chowdhury, M. H., G. Diamond, and L. K. Ryan. 2017. 11 Synergy of Antimicrobial Peptides. Antimicrobial Peptides: Discovery, Design Novel Therapeutic Strategies:pg 188.

Codjoe, F. S., and E. S. Donkor. 2018. Carbapenem resistance: a review. *Medical Sciences* **6**:1.

Collado, N., S. Rodriguez-Mozaz, M. Gros, A. Rubirola, D. Barcelo, J. Comas, I. Rodriguez-Roda, and G. Buttiglieri. 2014. Pharmaceuticals occurrence in a WWTP with significant industrial contribution and its input into the river system. *Environmental Pollution* **185**:202-212.

Commission, E. 2017. A European one health action plan against antimicrobial resistance (AMR). European Commission

Connor, R., A. Renata, C. Ortigara, E. Koncagül, S. Uhlenbrook, B. M. Lamizana-Diallo, S. M. Zadeh, M. Qadir, M. Kjellén, and J. Sjödin. 2017. The United Nations world water development report 2017. Wastewater: The untapped resource. The United Nations World Water Development Report.

Conte, D., J. K. Palmeiro, K. da Silva Nogueira, T. M. de Lima, M. A. Cardoso, R. Pontarolo, F. L. Degaut Pontes, and L. M. Dalla-Costa. 2017. Characterization of CTX-M enzymes, quinolone resistance determinants, and antimicrobial residues from hospital sewage, wastewater treatment plant, and river water. *Ecotoxicology and Environmental Safety* **136**:62-69.

Coque, T. M., F. Baquero, and R. Canton. 2008a. Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. *Euro Surveillance* **13**:19044.

Coque, T. M., A. Novais, A. Carattoli, L. Poirel, J. Pitout, L. Peixe, F. Baquero, R. Canton, and P. Nordmann. 2008b. Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum beta-lactamase CTX-M-15. *Emerging Infectious Diseases* **14**:195-200.

Cornaglia, G., H. Giamarellou, and G. M. Rossolini. 2011. Metallo-β-lactamases: a last frontier for β-lactams? *The Lancet infectious diseases* **11**:381-393.

Cycoń, M., A. Mrozik, and Z. Piotrowska-Seget. 2019. Antibiotics in the soil environment—degradation and their impact on microbial activity and diversity. *Frontiers in Microbiology* 10: 338.

Czekalski, N., T. Berthold, S. Caucci, A. Egli, and H. Bürgmann. 2012. Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. *Frontiers in Microbiology* **3**:106.

Czekalski, N., R. Sigdel, J. Birtel, B. Matthews, and H. Bürgmann. 2015. Does human activity impact the natural antibiotic resistance background? Abundance of antibiotic resistance genes in 21 Swiss lakes. *Environment international* **81**:45-55.

Da Costa, P. M., P. Vaz-Pires, and F. Bernardo. 2006. Antimicrobial resistance in *Enterococcus* spp. isolated in inflow, effluent and sludge from municipal sewage water treatment plants. *Water Research* **40**:1735-1740.

Daghrir, R., and P. Drogui. 2013. Tetracycline antibiotics in the environment: a review. *Environmental Chemistry Letters* **11**:209-227.

Damoa-Siakwan, S. 2005. Extended-spectrum beta lactamases: an overview. *British Journal of Infection Control* **6**:25-28.

Datta, N., and P. Kontomichalou. 1965. Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. *Nature* **208**:239-241.

Davies, J., and D. Davies. 2010. Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews* **74**:417-433.

Delcher, A. L., D. Harmon, S. Kasif, O. White, and S. L. Salzberg. 1999. Improved microbial gene identification with GLIMMER. *Nucleic Acids Research* **27**:4636-4641.

Deloger, M., M. El Karoui, and M.-A. s. Petit. 2009. A genomic distance based on MUM indicates discontinuity between most bacterial species and genera. *Journal of Bacteriology* **191**:91-99.

Deshpande, A., K. Baheti, and N. Chatterjee. 2004. Degradation of β-lactam antibiotics. *Current science*:1684-1695.

Dolejska, M., L. Villa, L. Poirel, P. Nordmann, and A. Carattoli. 2013. Complete sequencing of an IncHI1 plasmid encoding the carbapenemase NDM-1, the ArmA 16S RNA methylase and a resistance–nodulation–cell division/multidrug efflux pump. *Journal of Antimicrobial Chemotherapy* **68**:34-39.

Dong, H., X. Yuan, W. Wang, and Z. Qiang. 2016. Occurrence and removal of antibiotics in ecological and conventional wastewater treatment processes: A field study. *Journal of Environmental Management* **178**:11-19.

Drlica, K., H. Hiasa, R. Kerns, M. Malik, A. Mustaev, and X. Zhao. 2009. Quinolones: action and resistance updated. *Current Topics in Medicinal Chemistry* **9**:981-998.

ECDC. 2020. Annual Report of the European Antimicrobial Resistance Surveillance Network Antimicrobial resistance surveillance in Europe 2020. ECDC Stockholms.

Edelstein, M., M. Pimkin, I. Palagin, I. Edelstein, and L. Stratchounski. 2003. Prevalence and molecular epidemiology of CTX-M extended-spectrum beta-lactamase-producing *Escherichia coli* and Klebsiella pneumoniae in Russian hospitals. *Antimicrobial Agents and Chemotherapy* **47**:3724-3732.

Escobar-Zepeda, A., A. Vera-Ponce de Leon, and A. Sanchez-Flores. 2015. The Road to Metagenomics: From Microbiology to DNA Sequencing Technologies and Bioinformatics. *Frontiers in Genetics* **6**:348.

EUCAST. 2018. European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters. Version 8.1, 2018".

Felis, E., J. Kalka, A. Sochacki, K. Kowalska, S. Bajkacz, M. Harnisz, and E. Korzeniewska. 2020. Antimicrobial pharmaceuticals in the aquatic environment-occurrence and environmental implications. *European journal of pharmacology* **866**:172813.

Ferreira da Silva, M., I. Vaz-Moreira, M. Gonzalez-Pajuelo, O. C. Nunes, and C. M. Manaia. 2007. Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. *FEMS Microbiology Ecology* **60**:166-176.

Frase, H., Q. Shi, S. A. Testero, S. Mobashery, and S. B. Vakulenko. 2009. Mechanistic basis for the emergence of catalytic competence against carbapenem antibiotics by the GES family of beta-lactamases. *Journal of Biological Chemistry* **284**:29509-29513.

Gajdács, M. 2019. Epidemiology and resistance levels of Enterobacteriaceae isolates from urinary tract infections expressed as Multiple Antibiotic Resistance (MAR) indices. *Journal of Pharmaceutical Research International* **29**:: 7: 51055.

Galler, H., G. Feierl, C. Petternel, F. F. Reinthaler, D. Haas, A. J. Grisold, J. Luxner, and G. Zarfel. 2014. KPC-2 and OXA-48 carbapenemase-harbouring Enterobacteriaceae detected in an Austrian wastewater treatment plant. *Clinical Microbiology and Infection* **20**:O132-134.

Galvin, S., F. Boyle, P. Hickey, A. Vellinga, D. Morris, and M. Cormican. 2010. Enumeration and characterization of antimicrobial-resistant *Escherichia coli* bacteria in effluent from municipal, hospital, and secondary treatment facility sources. *Applied Environmental Microbiology* **76**:4772-4779.

Garcha, S., N. Verma, and S. Brar. 2016. Isolation, characterization and identification of microorganisms from unorganized dairy sector wastewater and sludge samples and evaluation of their biodegradability. *Water Resources Industry* **16**:19-28.

Gbylik-Sikorska, M., A. Posyniak, T. Sniegocki, and J. Zmudzki. 2015. Liquid chromatography–tandem mass spectrometry multiclass method for the determination of antibiotics residues

in water samples from water supply systems in food-producing animal farms. *Chemosphere* **119**:8-15.

Gelband, H., M. Miller-Petrie, P. Suraj, G. Sumanth, J. Levinson, D. Barter, A. White, and R. Laxminarayan. 2015. The state of the world's antibiotics. Centre for Diseases Dynamics, Economics and Policy, CDDEP,Washington D.C.

Genereux, D. P., and C. T. Bergstrom. 2005. Evolution in action: understanding antibiotic resistance. *Evolutionary Science*:145-153.

Göbel, A., A. Thomsen, C. S. McArdell, A. Joss, and W. Giger. 2005. Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment. *Environmental Science Technology* **39**:3981-3989.

Goldenman, G., M. Holland, J. Lietzmann, L. Meura, M. Camboni, A. Reihlen, and J. J. E. C. Bakker. 2017. Study for the strategy for a non-toxic environment of the 7th Environment Action Programme. European Commission.

Golovko, O., V. Kumar, G. Fedorova, T. Randak, and R. Grabic. 2014. Seasonal changes in antibiotics, antidepressants/psychiatric drugs, antihistamines and lipid regulators in a wastewater treatment plant. *Chemosphere* **111**:418-426.

González-Alonso, S., L. M. Merino, S. Esteban, M. L. de Alda, D. Barceló, J. J. Durán, J. López-Martínez, J. Aceña, S. Pérez, and N. Mastroianni. 2017. Occurrence of pharmaceutical, recreational and psychotropic drug residues in surface water on the northern Antarctic Peninsula region. *Environmental Pollution* **229**:241-254.

Götz, S., J. M. García-Gómez, J. Terol, T. D. Williams, S. H. Nagaraj, M. J. Nueda, M. Robles, M. Talón, J. Dopazo, and A. Conesa. 2008. High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Research* **36**:3420-3435.

Gracia-Lor, E., J. V. Sancho, R. Serrano, and F. Hernández. 2012. Occurrence and removal of pharmaceuticals in wastewater treatment plants at the Spanish Mediterranean area of Valencia. *Chemosphere* **87**:453-462.

Granato, E. T., C. Ziegenhain, R. L. Marvig, and R. Kummerli. 2018. Low spatial structure and selection against secreted virulence factors attenuates pathogenicity in *Pseudomonas aeruginosa*. *Multidisciplinary Journal of Microbial Ecology* **12**:2907-2918.

Gros, M., S. Rodríguez-Mozaz, and D. Barceló. 2013. Rapid analysis of multiclass antibiotic residues and some of their metabolites in hospital, urban wastewater and river water by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. *Journal of Chromatography* **1292**:173-188.

Gurung, M., D. C. Moon, M. D. Tamang, J. Kim, Y. C. Lee, S. Y. Seol, D. T. Cho, and J. C. Lee. 2010. Emergence of 16S rRNA methylase gene armA and cocarriage of blaIMP-1 in *Pseudomonas aeruginosa* isolates from South Korea. *Diagnostic microbiology infectious disease* **68**:468-470.

Haller, L., H. Chen, C. Ng, T. H. Le, T. H. Koh, T. Barkham, M. Sobsey, and K. Y.-H. Gin. 2018. Occurrence and characteristics of extended-spectrum β-lactamase-and carbapenemase-producing bacteria from hospital effluents in Singapore. *Science of the Total Environment* **615**:1119-1125.

Hanna, N., P. Sun, Q. Sun, X. Li, X. Yang, X. Ji, H. Zou, J. Ottoson, L. E. Nilsson, and B. Berglund. 2018. Presence of antibiotic residues in various environmental compartments of Shandong province in eastern China: its potential for resistance development and ecological and human risk. *Environment international* **114**:131-142.

Hansen, L. H., L. B. Jensen, H. I. Sorensen, and S. J. Sorensen. 2007. Substrate specificity of the OqxAB multidrug resistance pump in *Escherichia coli* and selected enteric bacteria. *Journal of Antimicrobial Chemotherapy* **60**:145-147.

Harrabi, M., S. V. Della Giustina, F. Aloulou, S. Rodriguez-Mozaz, D. Barceló, and B. Elleuch. 2018. Analysis of multiclass antibiotic residues in urban wastewater in Tunisia. *Environmental nanotechnology, monitoring* **10**:163-170.

Harris, S. J., M. Cormican, and E. Cummins. 2012. Antimicrobial residues and antimicrobial-resistant bacteria: impact on the microbial environment and risk to human health—a review. Human Ecological Risk Assessment: *An International Journal* **18**:767-809.

He, K., and L. Blaney. 2015. Systematic optimization of an SPE with HPLC-FLD method for fluoroquinolone detection in wastewater. *Journal of Hazardous Materials* **282**:96-105.

Hembach, N., F. Schmid, J. Alexander, C. Hiller, E. T. Rogall, and T. Schwartz. 2017. Occurrence of the mcr-1 colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany. *Frontiers in Microbiology* **8**:1282.

Hocquet, D., A. Muller, and X. Bertrand. 2016. What happens in hospitals does not stay in hospitals: antibiotic-resistant bacteria in hospital wastewater systems. *Journal of Hospital Infection* **93**:395-402.

Holten, K. B., and E. M. Onusko. 2000. Appropriate prescribing of oral beta-lactam antibiotics. *American Family Physician* **62**:611-620.

Hong, D. J., I. K. Bae, I. H. Jang, S. H. Jeong, H. K. Kang, and K. Lee. 2015. Epidemiology and Characteristics of Metallo-beta-Lactamase-Producing *Pseudomonas aeruginosa*. *Infection and Chemotherapy* **47**:81-97.

Hooper, L. V., and J. I. Gordon. 2001. Commensal host-bacterial relationships in the gut. *Science of the Total Environment* **292**:1115-1118.

Huson, D. H., and D. Bryant. 2006. Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* **23**:254-267.

Islam, M., M. Uddin, M. Hakim, K. Das, and M. Hasan. 2008. Role of untreated liquid hospital waste to the development of antibiotic resistant bacteria. *Journal of Innovation and Development Strategy* **2**:17-21.

Islam, Q. T. 2011. Antimicrobial resistance: a man made crisis. *Journal of Bangladesh College of Physicians Surgeons* **29**:120-125.

Islam, S., H. Oh, S. Jalal, F. Karpati, O. Ciofu, N. Hoiby, and B. Wretlind. 2009. Chromosomal mechanisms of aminoglycoside resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Clinical Microbiology and Infection* **15**:60-66.

Isozumi, R., K. Yoshimatsu, T. Yamashiro, F. Hasebe, B. M. Nguyen, T. C. Ngo, S. P. Yasuda, T. Koma, K. Shimizu, and J. Arikawa. 2012. blaNDM-1–positive *Klebsiella pneumoniae* from Environment, Vietnam. *Emerging Infectious Diseases* **18**:1383.

Ivone, V.-M., E. Conceição, N. Olga C, and M. Célia M. 2013. Bacterial diversity from the source to the tap: a comparative study based on 16S rRNA gene-DGGE and culture-dependent methods. *FEMS Microbiology Ecology* **83**:361-374.

Jacoby, G. A. 2005. Mechanisms of resistance to quinolones. *Clinical Infectious Diseases* **41 Suppl 2**:S120-126.

Jacoby, G. A. 2009. AmpC beta-lactamases. *Clinical Microbiology Reviews* **22**:161-182.

Jacoby, G. A., C. M. Griffin, and D. C. Hooper. 2011. *Citrobacter* spp. as a source of qnrB alleles. *Antimicrobial Agents* **55**:4979-4984.

Jacoby, G. A., and L. S. Munoz-Price. 2005. The new beta-lactamases. *New England Journal of Medicine* **352**:380-391.

Jin, J. S., K. T. Kwon, D. C. Moon, and J. C. Lee. 2009. Emergence of 16S rRNA methylase rmtA in colistin-only-sensitive *Pseudomonas aeruginosa* in South Korea. *International Journal of Antimicrobial Agents* **33**:490-491.

Johansen, H. K., S. M. Moskowitz, O. Ciofu, T. Pressler, and N. Høiby. 2008. Spread of colistin resistant non-mucoid *Pseudomonas aeruginosa* among chronically infected Danish cystic fibrosis patients. *Journal of Cystic Fibrosis* **7**:391-397.

Johnson, A. P., and N. Woodford. 2013. Global spread of antibiotic resistance: the example of New Delhi metallo-β-lactamase (NDM)-mediated carbapenem resistance. *Journal of Medical Microbiology* **62**:499-513.

Kaplan, E., N. Sela, A. Doron-Faigenboim, S. Navon-Venezia, E. Jurkevitch, and E. Cytryn. 2015. Genomic and Functional Characterization of qnr-Encoding Plasmids from Municipal Wastewater Biosolid *Klebsiella pneumoniae* Isolates. *Frontiers in Microbiology* **6**:1354.

Karkman, A., T. A. Johnson, C. Lyra, R. D. Stedtfeld, M. Tamminen, J. M. Tiedje, and M. Virta. 2016. High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant. *FEMS Microbiology Ecology* **92**:fiw014.

Kasprzyk-Hordern, B., R. M. Dinsdale, and A. J. Guwy. 2009. The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. *Water Research* **43**:363-380.

Kelly, B., A. Vespermann, D. J. F. Bolton, and C. Toxicology. 2009. Gene transfer events and their occurrence in selected environments. *Journal of Food and Chemical Toxicology* **47**:978-983.

Kemper, N. 2008. Veterinary antibiotics in the aquatic and terrestrial environment. *Ecological indicators* **8**:1-13.

Khan, I. U., and J. S. Yadav. 2004. Real-time PCR assays for genus-specific detection and quantification of culturable and non-culturable mycobacteria and pseudomonads in metalworking fluids. *Molecular and Cellular Probes* **18**:67-73.

Khuntayaporn, P., P. Montakantikul, P. Santanirand, P. Kiratisin, and M. T. Chomnawang. 2013. Molecular investigation of carbapenem resistance among multidrug-resistant *Pseudomonas aeruginosa* isolated clinically in Thailand. *Journal of Microbiology and Immunology* **57**:170-178.

Kim, S., J. N. Jensen, D. S. Aga, and A. S. Weber. 2007. Tetracycline as a selector for resistant bacteria in activated sludge. *Chemosphere* **66**:1643-1651.

King, L. J., L. R. Anderson, C. G. Blackmore, M. J. Blackwell, E. A. Lautner, L. C. Marcus, T. E. Meyer, T. P. Monath, J. E. Nave, and J. Ohle. 2008. Executive summary of the AVMA one health initiative task force report. *Journal of the American Veterinary Medical Association* **233**:259-261.

King, T. L. B., S. Schmidt, and S. Y. Essack. 2020. Antibiotic-resistant *Klebsiella* spp. from a hospital, hospital effluents and wastewater treatment plants in the uMgungundlovu District, KwaZulu-Natal, South Africa. *Science of the Total Environment* **712**:135550.

Kinney, C. A., and B. V. Heuvel. 2020. Translocation of pharmaceuticals and personal care products after land application of biosolids. *Current Opinion in Environmental Science and Health* **14**:23-30.

Koch, N., N. F. Islam, S. Sonowal, R. Prasad, and H. Sarma. 2021. Environmental antibiotics and resistance genes as emerging contaminants: methods of detection and bioremediation. *Current Research in Microbial Sciences* **2**:100027.

Köck, R., K. Becker, B. Cookson, J. van Gemert-Pijnen, S. Harbarth, J. Kluytmans, M. Mielke, G. Peters, R. Skov, and M. Struelens. 2010. Methicillin-resistant *Staphylococcus aureus (*MRSA): burden of disease and control challenges in Europe. *Euro Surveillance* **15**:19688.

Korzeniewska, E., and M. Harnisz. 2020. Sources, occurrence, and environmental risk assessment of antibiotics and antimicrobial-resistant bacteria in aquatic environments of Poland. Pages 179-193 Polish River Basins and Lakes–Part II. Springer.

Kosma, C. I., D. A. Lambropoulou, and T. A. Albanis. 2014. Investigation of PPCPs in wastewater treatment plants in Greece: occurrence, removal and environmental risk assessment. *Science of the Total Environment* **466**:421-438.

Kraemer, S. A., A. Ramachandran, and G. G. Perron. 2019. Antibiotic pollution in the environment: from microbial ecology to public policy. *Microorganisms* **7**:180.

Kumar, A., A. Patyal, and A. Panda. 2018. Sub-therapeutic use of antibiotics in animal feed and their potential impact on environmental and human health: a comprehensive review. *Animal Feed Science Technology* **6**:25.

Kumar, M., S. Jaiswal, K. K. Sodhi, P. Shree, D. K. Singh, P. K. Agrawal, and P. Shukla. 2019. Antibiotics bioremediation: Perspectives on its ecotoxicity and resistance. *Environment International* **124**:448-461.

Kummerer, K. 2004. Resistance in the environment. *Journal of Antimicrobial Chemotherapy* **54**:311-320.

Kümmerer, K. 2009. Antibiotics in the aquatic environment–a review–part I. *Chemosphere* **75**:417-434.

Kümmerer, K. 2009b. Antibiotics in the aquatic environment–a review–part I. *Chemosphere* **75**:417-434.

Lamba, M., D. W. Graham, and S. Z. Ahammad. 2017. Hospital Wastewater Releases of Carbapenem-Resistance Pathogens and Genes in Urban India. *Environmental Science and Technology* **51**:13906-13912.

Lara-Martín, P. A., E. González-Mazo, M. Petrovic, D. Barceló, and B. J. Brownawell. 2014. Occurrence, distribution and partitioning of nonionic surfactants and pharmaceuticals in the urbanized Long Island Sound Estuary (NY). *Marine Pollution Bulletin* **85**:710-719.

Lateef, A. 2004. The microbiology of a pharmaceutical effluent and its public health implications. *World Journal of Microbiology and Biotechnology* **20**:167-171.

Lautenbach, E., J. B. Patel, W. B. Bilker, P. H. Edelstein, and N. O. Fishman. 2001. Extended-spectrum beta-lactamase-producing Escherichia coli and *Klebsiella pneumoniae:* risk factors for infection and impact of resistance on outcomes. *Clinical Infectious Diseases* **32**:1162-1171.

Lettieri Teresa, S., Isabella, A. Navarro Cuenca, R. Loos, and D. Marinov. 2018. State of the Art on the Contribution of Water to Antimicrobial Resistance. Brussels: European Union.

Levy, S. B. 2002. The 2000 Garrod lecture. Factors impacting on the problem of antibiotic resistance. *Journal of Antimicrobial Chemotherapy* **49**:25-30.

Li, A. D., L. G. Li, and T. Zhang. 2015. Exploring antibiotic resistance genes and metal resistance genes in plasmid metagenomes from wastewater treatment plants. *Frontiers in Microbiology* **6**:1025.

Li, A. D., J. W. Metch, Y. Wang, E. Garner, A. N. Zhang, M. V. Riquelme, P. J. Vikesland, A. Pruden, and T. Zhang. 2018. Effects of sample preservation and DNA extraction on enumeration of antibiotic resistance genes in wastewater. *FEMS Microbiology Ecology* **94**:189.

Li, B., and T. Zhang. 2011. Mass flows and removal of antibiotics in two municipal wastewater treatment plants. *Chemosphere* **83**:1284-1289.

Li, W. C. 2014. Occurrence, sources, and fate of pharmaceuticals in aquatic environment and soil. *Environmental Pollution* **187**:193-201.

Libisch, B., M. Muzslay, M. Gacs, J. Minárovits, M. Knausz, J. Watine, G. Ternák, É. Kenéz, I. Kustos, and L. Rókusz. 2006. Molecular epidemiology of VIM-4 metallo-β-lactamase-producing *Pseudomonas* sp. isolates in Hungary. *Antimicrobial Agents and Chemotherapy* **50**:4220-4223.

Lin, A. Y., T. H. Yu, and S. K. Lateef. 2009. Removal of pharmaceuticals in secondary wastewater treatment processes in Taiwan. *Journal of Hazardous Materials* **167**:1163-1169.

Lincopan, N., P. Neves, E. M. Mamizuka, and C. E. Levy. 2010. Balanoposthitis caused by Pseudomonas aeruginosa co-producing metallo-β-lactamase and 16S rRNA methylase in children with hematological malignancies. *International Journal of Infectious Diseases* **14**:e344-e347.

Lindberg, R., P.-Å. Jarnheimer, B. Olsen, M. Johansson, and M. Tysklind. 2004. Determination of antibiotic substances in hospital sewage water using solid phase extraction and liquid chromatography/mass spectrometry and group analogue internal standards. *Chemosphere* **57**:1479-1488.

Lindberg, R. H., K. Bjorklund, P. Rendahl, M. I. Johansson, M. Tysklind, and B. A. Andersson. 2007. Environmental risk assessment of antibiotics in the Swedish environment with emphasis on sewage treatment plants. *Water Research* **41**:613-619.

Lindberg, R. H., U. Olofsson, P. Rendahl, M. I. Johansson, M. Tysklind, and B. A. Andersson. 2006. Behavior of fluoroquinolones and trimethoprim during mechanical, chemical, and active sludge treatment of sewage water and digestion of sludge. *Environmental Science and Technology* **40**:1042-1048.

Lister, P. D., D. J. Wolter, and N. D. Hanson. 2009. Antibacterial-resistant *Pseudomonas aeruginosa:* clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clinical Microbiology Reviews* **22**:582-610.

Liu, J., P. Keelan, P. M. Bennett, and V. I. Enne. 2009. Characterization of a novel macrolide efflux gene, mef (B), found linked to sul3 in porcine *Escherichia coli*. *Antimicrobial Chemotherapy* **63**:423-426.

Livermore, D. M., and P. M. Hawkey. 2005. CTX-M: changing the face of ESBLs in the UK. *Journal of Antimicrobial Chemotherapy* **56**:451-454.

Löffler, D., and T. A. Ternes. 2003. Analytical method for the determination of the aminoglycoside gentamicin in hospital wastewater via liquid chromatography–electrospray-tandem mass spectrometry. *Chromatography* A **1000**:583-588.

Loos, R., R. Carvalho, D. C. Antonio, S. Comero, G. Locoro, S. Tavazzi, B. Paracchini, M. Ghiani, T. Lettieri, L. Blaha, B. Jarosova, S. Voorspoels, K. Servaes, P. Haglund, J. Fick, R. H. Lindberg, D. Schwesig, and B. M. Gawlik. 2013. EU-wide monitoring survey on emerging

polar organic contaminants in wastewater treatment plant effluents. *Water Research* **47**:6475-6487.

Loos, R., D. Marinov, I. Sanseverino, D. Napierska, and T. Lettieri. 2018. Review of the 1st Watch List under the Water Framework Directive and recommendations for the 2nd Watch List. Luxembourg: Publications Office of the European Union.

Lorenzo, P., A. Adriana, S. Jessica, B. Carles, F. Marinella, L. Marta, B. J. Luis, and S. Pierre. 2018. Antibiotic resistance in urban and hospital wastewaters and their impact on a receiving freshwater ecosystem. *Chemosphere* **206**:70-82.

Lupo, A., S. Coyne, and T. U. Berendonk. 2012. Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. *Frontiers in microbiology* **3**:18.

MacDougall, C. 2011. Beyond susceptible and resistant, part I: treatment of infections due to Gram-negative organisms with inducible β-lactamases. *The Journal of Pediatric Pharmacology Therapeutics* **16**:23-30.

Mackenzie, J. S., P. Drury, R. R. Arthur, M. J. Ryan, T. Grein, R. Slattery, S. Suri, C. T. Domingo, and A. Bejtullahu. 2014. The global outbreak alert and response network. *Global Public Health* **9**:1023-1039.

Magyar, A., B. Köves, K. Nagy, A. Dobák, V. K. A. Arthanareeswaran, P. Bálint, F. Wagenlehner, and P. Tenke. 2017. Spectrum and antibiotic resistance of uropathogens between 2004 and 2015 in a tertiary care hospital in Hungary. *Journal of Medical Microbiology* **66**:788-797.

Managaki, S., A. Murata, H. Takada, B. C. Tuyen, and N. H. Chiem. 2007. Distribution of macrolides, sulfonamides, and trimethoprim in tropical waters: ubiquitous occurrence of veterinary antibiotics in the Mekong Delta. *Environmental Science and Technology* **41**:8004-8010.

Manzetti, S., and R. Ghisi. 2014. The environmental release and fate of antibiotics. *Marine Pollution Bulletin* **79**:7-15.

Marçais, G., A. L. Delcher, A. M. Phillippy, R. Coston, S. L. Salzberg, and A. Zimin. 2018. MUMmer4: A fast and versatile genome alignment system. *Computational Biology* **14**:e1005944.

Marchiaro, P., V. Ballerini, T. Spalding, G. Cera, M. A. Mussi, J. Moran-Barrio, A. J. Vila, A. M. Viale, and A. S. Limansky. 2008. A convenient microbiological assay employing cell-free extracts for the rapid characterization of Gram-negative carbapenemase producers. *Journal of Antimicrobial Chemotherapy* **62**:336-344.

Martínez-Martínez, L. 2008. Extended-spectrum β-lactamases and the permeability barrier. *Clinical Microbiology Infection Control* **14**:82-89.

Matongo, S., G. Birungi, B. Moodley, and P. Ndungu. 2015. Occurrence of selected pharmaceuticals in water and sediment of Umgeni River, KwaZulu-Natal, South Africa. *Environmental Science and Pollution Research International* **22**:10298-10308.

McCarthy, A., E. Chiang, M. L. Schmidt, and V. J. Denef. 2015. RNA preservation agents and nucleic acid extraction method bias perceived bacterial community composition. *PLoS One* **10**:e0121659.

McLain, J. E., E. Cytryn, L. M. Durso, and S. Young. 2016. Culture-based Methods for Detection of Antibiotic Resistance in Agroecosystems: Advantages, Challenges, and Gaps in Knowledge. *Journal of Environmental Quality* **45**:432-440.

Melegh, S., K. Kovács, T. Gám, A. Nyul, B. Patkó, A. Tóth, I. Damjanova, and G. Mestyán. 2014a. Emergence of VIM-4 metallo-β-lactamase-producing *Klebsiella pneumoniae* ST15 clone

in the Clinical Centre University of Pécs, Hungary. *Clinical Microbiology Infection Control* **20**:O27-O29.

Melegh, S., K. Kovács, T. Gám, A. Nyul, B. Patkó, A. Tóth, I. Damjanova, G. J. C. M. Mestyán, and Infection. 2014b. Emergence of VIM-4 metallo-β-lactamase-producing *Klebsiella pneumoniae* ST15 clone in the Clinical Centre University of Pécs, Hungary. *Journal of Antimicrobial Chemotherapy* **20**:O27-O29.

Memar, M. Y., K. Adibkia, S. Farajnia, H. S. Kafil, M. Yekani, N. Alizadeh, and R. Ghotaslou. 2019. The grape seed extract: a natural antimicrobial agent against different pathogens. *Reviews in Medical Microbiology* **30**:173-182.

Mendoza, A., J. Aceña, S. Pérez, M. L. De Alda, D. Barceló, A. Gil, and Y. Valcárcel. 2015. Pharmaceuticals and iodinated contrast media in a hospital wastewater: a case study to analyse their presence and characterise their environmental risk and hazard. *Environmental Research* **140**:225-241.

Michael, I., L. Rizzo, C. McArdell, C. Manaia, C. Merlin, T. Schwartz, C. Dagot, and D. Fatta-Kassinos. 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review. *Water Research* **47**:957-995.

Minh, T. B., H. W. Leung, I. H. Loi, W. H. Chan, M. K. So, J. Q. Mao, D. Choi, J. C. Lam, G. Zheng, M. Martin, J. H. Lee, P. K. Lam, and B. J. Richardson. 2009. Antibiotics in the Hong Kong metropolitan area: Ubiquitous distribution and fate in Victoria Harbour. *Marine Pollution Bulletin* **58**:1052-1062.

Moore, D. F., J. A. Guzman, and C. McGee. 2008. Species distribution and antimicrobial resistance of enterococci isolated from surface and ocean water. *Journal of Applied Microbiology* **105**:1017-1025.

Moran, R. A., S. Anantham, J. L. Pinyon, and R. M. Hall. 2015. Plasmids in antibiotic susceptible and antibiotic resistant commensal *Escherichia coli* from healthy Australian adults. *Plasmid* **80**:24-31.

Moreno-González, R., S. Rodríguez-Mozaz, M. Gros, E. Pérez-Cánovas, D. Barceló, and V. M. León. 2014. Input of pharmaceuticals through coastal surface watercourses into a Mediterranean lagoon (Mar Menor, SE Spain): sources and seasonal variations. *Science of the Total Environment* **490**:59-72.

Morris, D., S. Harris, C. Morris, E. Commins, and M. Cormican. 2008. Hospital effluent: impact on the microbial environment and risk to human health. Environment protection agency. Ireland.

Morris, D., S. Harris, C. Morris, E. Commins, and M. Cormican. 2016. Report no. 162 hospital effluent: impact on the microbial environment and risk to human health.

Munck, C., M. Albertsen, A. Telke, M. Ellabaan, P. H. Nielsen, and M. O. A. Sommer. 2015. Limited dissemination of the wastewater treatment plant core resistome. *Nature Communications* **6**:8452.

Muramatsu, H., T. Horii, A. Takeshita, H. Hashimoto, and M. Maekawa. 2005. Characterization of fluoroquinolone and carbapenem susceptibilities in clinical isolates of levofloxacin-resistant *Pseudomonas aeruginosa*. *Chemotherapy* **51**:70-75.

Mutuku, C., Z. Gazdag, and S. J. W. J. o. M. Melegh. 2022. Occurrence of antibiotics and bacterial resistance genes in wastewater: Resistance mechanisms and antimicrobial resistance control approaches. *World Journal of Microbiology and Biotechnology* **38**:1-27.

Nagy, B. J., B. Balazs, I. Benmazouz, P. Gyure, L. Kover, E. Kaszab, K. Bali, A. Lovas-Kiss, I. Damjanova, L. Majoros, A. Toth, K. Banyai, and G. Kardos. 2021. Comparison of Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli* Isolates From Rooks (Corvus frugilegus) and Contemporary Human-Derived Strains: A One Health Perspective. *Frontiers in Microbiology* **12**:785411.

Narayanan, N., L. Johnson, and C. MacDougall. 2016. Beyond susceptible and resistant, part III: treatment of infections due to gram-negative organisms producing carbapenemases. *The Journal of Pediatric Pharmacology Therapeutics* **21**:110-119.

Navon-Venezia, S., R. Ben-Ami, and Y. Carmeli. 2005. Update on *Pseudomonas aeruginosa* and Acinetobacter baumannii infections in the healthcare setting. *Current Opinion in Infectious Diseases* **18**:306-313.

Ngumba, E., A. Gachanja, and T. Tuhkanen. 2016. Occurrence of selected antibiotics and antiretroviral drugs in Nairobi River Basin, Kenya. *Science of the Total Environment* **539**:206-213.

Nguyen, L., J. Garcia, K. Gruenberg, and C. MacDougall. 2018. Multidrug-Resistant *Pseudomonas* Infections: Hard to Treat, But Hope on the Horizon? *Current Infectious Disease Reports* **20**(8):23

Nnadozie, C., S. Kumari, and F. Bux. 2017. Status of pathogens, antibiotic resistance genes and antibiotic residues in wastewater treatment systems. *Reviews in Environmental Science Bio/Technology* **16**:491-515.

Nordmann Guibert, M. 1998. Extended spectrum β-lactamases. *Antimicrobial Chemotherapy* **42**:128-131.

Nordmann, P., G. Cuzon, and T. Naas. 2009. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *The Lancet infectious diseases* **9**:228-236.

Nordmann, P., and L. Poirel. 2002. Emerging carbapenemases in Gram-negative aerobes. *Clinical Microbiology and Infection* **8**:321-331.

Novo, A., and C. M. Manaia. 2010. Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. *Applied Microbiology and Biotechnology* **87**:1157-1166.

Oberlé, K., M.-J. Capdeville, T. Berthe, H. l. n. Budzinski, F. e. Petit, and technology. 2012. Evidence for a complex relationship between antibiotics and antibiotic-resistant *Escherichia coli*: from medical center patients to a receiving environment. *Journal of Environmental Science* **46**:1859-1868.

Odjadjare, E. E., E. O. Igbinosa, R. Mordi, B. Igere, C. L. Igeleke, and A. I. Okoh. 2012. Prevalence of multiple antibiotics resistant (MAR) *Pseudomonas* spp. in the final effluents of three municipal wastewater treatment facilities in South Africa. *International Journal of Environmental Research and Public Healt*h **9**:2092-2107.

Oduro-Mensah, D., N. Obeng-Nkrumah, E. Y. Bonney, E. Oduro-Mensah, K. Twum-Danso, Y. D. Osei, and S. T. Sackey. 2016. Genetic characterization of TEM-type ESBL-associated antibacterial resistance in Enterobacteriaceae in a tertiary hospital in Ghana. *Annals of Clinical Microbiology and Antimicrobials* **15**:29.

Oie, S., Y. Fukui, M. Yamamoto, Y. Masuda, and A. Kamiya. 2009. In vitro antimicrobial effects of aztreonam, colistin, and the 3-drug combination of aztreonam, ceftazidime and amikacin on metallo-β-lactamase-producing *Pseudomonas aeruginosa*. *BMC Infectious Diseases* **9**:1-5.

Ojer-Usoz, E., D. Gonzalez, I. Garcia-Jalon, and A. I. Vitas. 2014a. High dissemination of extended-spectrum beta-lactamase-producing Enterobacteriaceae in effluents from wastewater treatment plants. *Water Research* **56**:37-47.

Ojer-Usoz, E., D. González, I. García-Jalón, and A. I. Vitas. 2014b. High dissemination of extended-spectrum β-lactamase-producing Enterobacteriaceae in effluents from wastewater treatment plants. *Water Research* **56**:37-47.

Olesen, I., H. Hasman, and F. Møller Aarestrup. 2004. Prevalence of β-lactamases among ampicillin-resistant *Escherichia coli* and *Salmonella* isolated from food animals in Denmark. *Microbial Drug Resistance* **10**:334-340.

Opriş, O., M.-L. Soran, V. Coman, F. Copaciu, and D. Ristoiu. 2013. Determination of some frequently used antibiotics in waste waters using solid phase extraction followed by high performance liquid chromatography with diode array and mass spectrometry detection. *Central European Journal of Chemistry* **11**:1343-1351.

Osorio, V., R. Marcé, S. Pérez, A. Ginebreda, J. L. Cortina, and D. Barceló. 2012. Occurrence and modeling of pharmaceuticals on a sewage-impacted Mediterranean river and their dynamics under different hydrological conditions. *Science of the Total Environment* **440**:3-13.

Östman, M., R. H. Lindberg, J. Fick, E. Björn, and M. Tysklind. 2017. Screening of biocides, metals and antibiotics in Swedish sewage sludge and wastewater. *Water Research* **115**:318-328.

Osundiya, O., R. Oladele, and O. Oduyebo. 2013. Multiple antibiotic resistance (MAR) indices of *Pseudomonas* and *Klebsiella* species isolates in Lagos University Teaching Hospital. *African Journal of Clinical Experimental Microbiology* **14**:164-168.

Oulas, A., C. Pavloudi, P. Polymenakou, G. A. Pavlopoulos, N. Papanikolaou, G. Kotoulas, C. Arvanitidis, and I. Iliopoulos. 2015. Metagenomics: tools and insights for analyzing next-generation sequencing data derived from biodiversity studies. *Bioinformatics and Biology Insights* **9**:75-88.

Pai, H., M. R. Kim, M. R. Seo, T. Y. Choi, and S. H. Oh. 2006. A nosocomial outbreak of *Escherichia coli* producing CTX-M-15 and OXA-30 beta-lactamase. *Infection Control and Hospital Epidemiology* **27**:312-314.

Pang, Z., R. Raudonis, B. R. Glick, T.-J. Lin, and Z. Cheng. 2019. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnology Advances* **37**:177-192.

Papageorgiou, M., C. Kosma, and D. Lambropoulou. 2016. Seasonal occurrence, removal, mass loading and environmental risk assessment of 55 pharmaceuticals and personal care products in a municipal wastewater treatment plant in Central Greece. *Science of the Total Environment* **543**:547-569.

Parkins, M. D., R. Somayaji, and V. J. Waters. 2018. Epidemiology, Biology, and Impact of Clonal *Pseudomonas aeruginosa* Infections in Cystic Fibrosis. *Clinical Microbiology Reviews* **31**.

Pärnänen, K. M., C. Narciso-da-Rocha, D. Kneis, T. U. Berendonk, D. Cacace, T. T. Do, C. Elpers, D. Fatta-Kassinos, I. Henriques, and T. J. S. a. Jaeger. 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. *Science Advances* **5**:9124.

Partridge, S. R. 2011. Analysis of antibiotic resistance regions in Gram-negative bacteria. *FEMS Microbiology Reviews* **35**:820-855.

Partridge, S. R. 2015. Resistance mechanisms in Enterobacteriaceae. *Pathology* **47**:276-284.

Partridge, S. R., G. Tsafnat, E. Coiera, and J. R. Iredell. 2009a. Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiology Reviews* **33**:757-784.

Partridge, S. R., G. Tsafnat, E. Coiera, and J. R. Iredell. 2009b. Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiology Reviews* **33**:757-784.

Paterson, D. L., and R. A. Bonomo. 2005. Extended-spectrum beta-lactamases: a clinical update. *Clinical Microbiology Reviews* **18**:657-686.

Paul, D., D. Dhar Chanda, A. P. Maurya, S. Mishra, A. Chakravarty, G. D. Sharma, and A. Bhattacharjee. 2015. Co-Carriage of bla KPC-2 and bla NDM-1 in Clinical Isolates of *Pseudomonas aeruginosa* Associated with Hospital Infections from India. *PLoS One* **10**:145823.

Penalva, G., L. D. Hogberg, K. Weist, V. Vlahovic-Palcevski, O. Heuer, D. L. Monnet, E. Vandael, I. N. Ivanov, M. Payerl-Pal, K. Skjold Selle Pedersen, E. Linask, B. Schweickert, F. Kontopidou, M. Matuz, E. Sarvikivi, P. Cavalie, A. Oza, G. Sigmundsdottir, F. Fortinguerra, R. Valinteliene, M. Bruch, P. Zarb, S. Natsch, H. S. Blix, A. Olczak-Pienkowska, I. Iosif, M. T. Alonso Herreras, V. Wiener, M. Cizman, T. Tesar, B. Muller-Pebody, L. Catteau, S. D. Sabtcheva, A. Tambic Andrasevic, P. Maikanti-Charalampous, H. Zemlickova, U. Sonksen, M. Ivanova, S. Maugat, J. Jalava, I. Noll, M. Polemis, Z. Vegh, K. G. Kristinsson, K. Burns, F. D'Ancona, K. Vulane, J. Miciuleviciene, M. Perrin, E. A. Scicluna, A. F. Schoffelen, G. S. Simonsen, D. Zabicka, M. Canica, G. A. Popescu, E. Schreterova, H. Ribic, B. Aracil, H. Billstrom, J. Wilson, E.-N. S. Grp, and E.-N. S. Grp. 2019. Decreasing and stabilising trends of antimicrobial consumption and resistance in *Escherichia coli* and *Klebsiella pneumoniae* in segmented regression analysis, European Union/European Economic Area, 2001 to 2018. *Euro Surveillance* **24**:2-8.

Petrovic, M., M. Gros, and D. Barcelo. 2006. Multi-residue analysis of pharmaceuticals in wastewater by ultra-performance liquid chromatography–quadrupole–time-of-flight mass spectrometry. *Chromatography A* **1124**:68-81.

Picão, R. C., L. Poirel, A. C. Gales, and P. Nordmann. 2009. Further identification of CTX-M-2 extended-spectrum β-lactamase in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **53**:2225-2226.

Pitout, J. D., and K. B. Laupland. 2008. Extended-spectrum β-lactamase-producing Enterobacteriaceae: an emerging public-health concern. *The Lancet Infectious Diseases* **8**:159-166.

Poirel, L., J. Y. Madec, A. Lupo, A. K. Schink, N. Kieffer, P. Nordmann, and S. Schwarz. 2018. Antimicrobial Resistance in *Escherichia coli*. *Microbiology Spectrum* **6**:6.4. 14.

Poirel, L., A. Potron, and P. Nordmann. 2012. OXA-48-like carbapenemases: the phantom menace. *Journal of Antimicrobal Chemotherapy* **67**:1597-1606.

Poirel, L., J. M. Rodriguez-Martinez, H. Mammeri, A. Liard, and P. Nordmann. 2005. Origin of plasmid-mediated quinolone resistance determinant QnrA. *Antimicrobial Agents and Chemotherapy* **49**:3523-3525.

Poirel, L., T. R. Walsh, V. Cuvillier, and P. Nordmann. 2011. Multiplex PCR for detection of acquired carbapenemase genes. *Diagnostic Microbiology and Infectious Diseases* **70**:119-123.

Poole, K. 2000. Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. *Antimicrobial Agents and Chemotherapy* **44**:2233-2241.

Poole, K. 2011. *Pseudomonas aeruginosa*: resistance to the max. *Frontiers in Microbiology* **2**:65.

Potron, A., L. Poirel, and P. Nordmann. 2015. Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: Mechanisms and epidemiology. *International Journal of Antimicrobial Agents* **45**:568-585.

Prado, T., W. C. Pereira, D. M. Silva, L. M. Seki, A. P. Carvalho, and M. D. Asensi. 2008. Detection of extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* in effluents and sludge of a hospital sewage treatment plant. *Letters in Applied Microbiology* **46**:136-141.

Prescott, J. F. 2013. Sulfonamides, diaminopyrimidines, and their combinations. *Antimicrobial Therapy in Veterinary Medicine* **5**:279-294.

Qin, X., D. M. Zerr, S. J. Weissman, J. A. Englund, D. M. Denno, E. J. Klein, P. I. Tarr, J. Kwong, J. R. Stapp, L. G. Tulloch, and E. Galanakis. 2008. Prevalence and mechanisms of broad-spectrum beta-lactam resistance in Enterobacteriaceae: a children's hospital experience. *Antimicrobial Agents and Chemotherapy* **52**:3909-3914.

Rabbani, M. A. G., M. Z. H. Howlader, and Y. Kabir. 2017. Detection of multidrug resistant (MDR) bacteria in untreated waste water disposals of hospitals in Dhaka City, Bangladesh. *Journal of Global Antimicrobial Resistance* **10**:120-125.

Ramirez, M. S., and M. E. Tolmasky. 2010. Aminoglycoside modifying enzymes. *Drug Resistance Updates* **13**:151-171.

Rasmussen, B. A., and K. Bush. 1997. Carbapenem-hydrolyzing beta-lactamases. *Antimicrobial Agents and Chemotherapy* **41**:223-232.

Recchia, G. D., and R. M. Hall. 1995. Gene cassettes: a new class of mobile element. *Microbiology (Reading)* **141 (Pt 12)**:3015-3027.

Reinhardt, A., T. Kohler, P. Wood, P. Rohner, J. L. Dumas, B. Ricou, and C. van Delden. 2007. Development and persistence of antimicrobial resistance in *Pseudomonas aeruginosa*: a longitudinal observation in mechanically ventilated patients. *Antimicrobial Agents and Chemotherapy* **51**:1341-1350.

Reis, E. O., A. F. S. Foureaux, J. S. Rodrigues, V. R. Moreira, Y. A. Lebron, L. V. Santos, M. C. Amaral, and L. C. Lange. 2019. Occurrence, removal and seasonal variation of pharmaceuticals in Brasilian drinking water treatment plants. *Environmental Pollution* **250**:773-781.

Rejiba, S., A. Aubry, S. Petitfrere, V. Jarlier, and E. Cambau. 2008. Contribution of ParE mutation and efflux to ciprofloxacin resistance in *Pseudomonas aeruginosa* clinical isolates. *Chemotherapy* **20**:749-752.

Riaz, L., T. Mahmood, A. Khalid, A. Rashid, M. B. A. Siddique, A. Kamal, and M. S. Coyne. 2018. Fluoroquinolones (FQs) in the environment: a review on their abundance, sorption and toxicity in soil. *Chemosphere* **191**:704-720.

Ribeiro, A. R., B. Sures, and T. C. Schmidt. 2018. Cephalosporin antibiotics in the aquatic environment: A critical review of occurrence, fate, ecotoxicity and removal technologies. *Environmental Pollution* **241**:1153-1166.

Ricci, M., R. Lava, and B. Koleva. 2016. Matrix certified reference materials for environmental monitoring under the EU water framework directive: an update. *Trends in Analytical Chemistry* **76**:194-202.

Rizzo, L., C. Manaia, C. Merlin, T. Schwartz, C. Dagot, M. Ploy, I. Michael, and D. Fatta-Kassinos. 2013a. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Science of the Total Environment* **447**:345-360.

Rizzo, L., C. Manaia, C. Merlin, T. Schwartz, C. Dagot, M. Ploy, I. Michael, and D. Fatta-Kassinos. 2013b. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Science of the Total Environment* **447**:345-360.

Robicsek, A., G. A. Jacoby, and D. C. Hooper. 2006a. The worldwide emergence of plasmid-mediated quinolone resistance. *The Lancet Infectious Diseases* **6**:629-640.

Robicsek, A., J. Strahilevitz, G. A. Jacoby, M. Macielag, D. Abbanat, C. H. Park, K. Bush, and D. C. Hooper. 2006b. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nature Medicine* **12**:83-88.

Rocha, J., D. Cacace, I. Kampouris, H. Guilloteau, T. Jäger, R. B. Marano, P. Karaolia, C. M. Manaia, C. Merlin, and D. Fatta-Kassinos. 2020. Inter-laboratory calibration of quantitative analyses of antibiotic resistance genes. *Environmental Chemical Engineering* **8**:102214.

Rodríguez-Martínez, J.-M., L. Poirel, and P. Nordmann. 2009. Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **53**:4783-4788.

Rossmann, J., S. Schubert, R. Gurke, R. Oertel, and W. Kirch. 2014. Simultaneous determination of most prescribed antibiotics in multiple urban wastewater by SPE-LC–MS/MS. *Journal of Chromatography B* **969**:162-170.

Rozman, U., D. Duh, M. Cimerman, and S. Š. Turk. 2020. Hospital wastewater effluent: hot spot for antibiotic resistant bacteria. *Journal of Water, Sanitation Hygiene for Development* **10**:171-178.

Rozwandowicz, M., M. S. M. Brouwer, J. Fischer, J. A. Wagenaar, B. Gonzalez-Zorn, B. Guerra, D. J. Mevius, and J. Hordijk. 2018. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. *Antimicrobial Chemotherapy* **73**:1121-1137.

Ruff, M., M. S. Mueller, M. Loos, and H. P. Singer. 2015. Quantitative target and systematic non-target analysis of polar organic micro-pollutants along the river Rhine using high-resolution mass-spectrometry–Identification of unknown sources and compounds. *Water Research* **87**:145-154.

Ruppé, É., P.-L. Woerther, and F. Barbier. 2015. Mechanisms of antimicrobial resistance in Gram-negative bacilli. *Annals of Intensive Care* **5**:1-15.

Sabino, Y. N. V., M. F. Santana, L. B. Oyama, F. G. Santos, A. J. S. Moreira, S. A. Huws, and H. C. Mantovani. 2019. Characterization of antibiotic resistance genes in the species of the rumen microbiota. *Nature Communications* **10**:5252.

Santajit, S., and N. Indrawattana. 2016. Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. *BioMed Research International* **2016**:2475067.

Santos, L. H., M. Gros, S. Rodriguez-Mozaz, C. Delerue-Matos, A. Pena, D. Barcelo, and M. C. Montenegro. 2013. Contribution of hospital effluents to the load of pharmaceuticals in urban wastewaters: identification of ecologically relevant pharmaceuticals. *Science of the Total Environment* **461-462**:302-316.

Schluter, A., R. Szczepanowski, A. Puhler, and E. M. Top. 2007. Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiology Reviews* **31**:449-477.

Schwartz, T. 2012. Strategies to assess and minimize the biological risk of antibiotic resistance in the environment. John Wiley & Sons: Hoboken, NJ, USA.

Seifrtova, M., L. Novakova, C. Lino, A. Pena, and P. Solich. 2009. An overview of analytical methodologies for the determination of antibiotics in environmental waters. *Analitica Chimica Acta* **649**:158-179.

Sghaier, S., M. S. Abbassi, A. Pascual, L. Serrano, P. Diaz-De-Alba, M. B. Said, B. Hassen, C. Ibrahim, A. Hassen, and L. Lopez-Cerero. 2019. Extended-spectrum beta-lactamase-producing Enterobacteriaceae from animal origin and wastewater in Tunisia: first detection of O25b-B23-CTX-M-27-ST131 *Escherichia coli* and CTX-M-15/OXA-204-producing *Citrobacter freundii* from wastewater. *Global Antimicrobial Resistance* **17**:189-194.

Sheikh, A. F., S. Rostami, A. Jolodar, M. A. Tabatabaiefar, F. Khorvash, A. Saki, S. Shoja, and R. Sheikhi. 2014. Detection of metallo-beta lactamases among carbapenem-resistant *Pseudomonas aeruginosa*. *Jundishapur Journal of Microbiology* **7**.

Sidstedt, M., L. Jansson, E. Nilsson, L. Noppa, M. Forsman, P. Rådström, and J. Hedman. 2015. Humic substances cause fluorescence inhibition in real-time polymerase chain reaction. *Analytical Biochemistry* **487**:30-37.

Siqueira, A. K., G. B. Michael, D. F. Domingos, M. M. Ferraz, M. G. Ribeiro, S. Schwarz, and D. S. Leite. 2016. Diversity of class 1 and 2 integrons detected in *Escherichia coli* isolates from diseased and apparently healthy dogs. *Veterinary Microbiology* **194**:79-83.

Slama, T. G. 2008. Gram-negative antibiotic resistance: there is a price to pay. *Critical Care* **12**:S4.

Slekovec, C., J. Plantin, P. Cholley, M. Thouverez, D. Talon, X. Bertrand, and D. Hocquet. 2012. Tracking down antibiotic-resistant *Pseudomonas aeruginosa* isolates in a wastewater network. *PLoS One* **7**:e49300.

Smet, A., A. Martel, D. Persoons, J. Dewulf, M. Heyndrickx, B. Catry, L. Herman, F. Haesebrouck, and P. Butaye. 2008. Diversity of extended-spectrum β-lactamases and class C β-lactamases among cloacal *Escherichia coli* isolates in Belgian broiler farms. *Antimicrobial Agents and Chemotherapy* **52**:1238-1243.

Spongberg, A. L., and J. D. Witter. 2008. Pharmaceutical compounds in the wastewater process stream in Northwest Ohio. *Science of the Total Environment* **397**:148-157.

Stephens, C., T. Arismendi, M. Wright, A. Hartman, A. Gonzalez, M. Gill, M. Pandori, and D. Hess. 2020. F Plasmids Are the Major Carriers of Antibiotic Resistance Genes in Human-Associated Commensal *Escherichia coli*. *Msphere* **5**:00709-00720.

Strahilevitz, J., G. A. Jacoby, D. C. Hooper, and A. Robicsek. 2009. Plasmid-mediated quinolone resistance: a multifaceted threat. *Clinical Microbiology Reviews* **22**:664-689.

Strateva, T., and D. Yordanov. 2009. *Pseudomonas aeruginosa*–a phenomenon of bacterial resistance. *Journal of Medical Microbiology* **58**:1133-1148.

Sunde, M., H. Solheim, and J. S. Slettemeas. 2008. Genetic linkage between class 1 integrons with the dfrA12-orfF-aadA2 cassette array and sul3 in *Escherichia coli*. *Veterinary Microbiology* **130**:422-425.

Sykes, R. B. B., Karen 1982. Physiology, biochemistry, and inactivation of β-lactamases. Pages 155-207 The Biology of Beta-Lactam Antibiotics. Elsevier.

Szczepanowski, R., B. Linke, I. Krahn, K. H. Gartemann, T. Gutzkow, W. Eichler, A. Puhler, and A. Schluter. 2009. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology (Reading)* **155**:2306-2319.

Szymańska, U., M. Wiergowski, I. Sołtyszewski, J. Kuzemko, G. Wiergowska, and M. K. Woźniak. 2019. Presence of antibiotics in the aquatic environment in Europe and their

analytical monitoring: recent trends and perspectives. *Microchemical Journal* **147**:729-740.

Tacconelli, E., E. Carrara, A. Savoldi, S. Harbarth, M. Mendelson, D. L. Monnet, C. Pulcini, G. Kahlmeter, J. Kluytmans, Y. Carmeli, M. Ouellette, K. Outterson, J. Patel, M. Cavaleri, E. M. Cox, C. R. Houchens, M. L. Grayson, P. Hansen, N. Singh, U. Theuretzbacher, N. Magrini, and W. H. O. P. P. L. W. Group. 2018. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *The Lancet Infectious Diseases* **18**:318-327.

Tahrani, L., J. Van Loco, H. Ben Mansour, and T. Reyns. 2016. Occurrence of antibiotics in pharmaceutical industrial wastewater, wastewater treatment plant and sea waters in Tunisia. *Journal of Water Health Sciences* **14**:208-213.

Talaro, K. P. 2017. Foundations in microbiology: basic principles. MCGRAW-HILL US HIGHER ED.

Tam, V. H., K. T. Chang, K. Abdelraouf, C. G. Brioso, M. Ameka, L. A. McCaskey, J. S. Weston, J. P. Caeiro, and K. W. Garey. 2010. Prevalence, resistance mechanisms, and susceptibility of multidrug-resistant bloodstream isolates of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **54**:1160-1164.

Tang, S. S., A. Apisarnthanarak, and L. Y. Hsu. 2014. Mechanisms of beta-lactam antimicrobial resistance and epidemiology of major community- and healthcare-associated multidrug-resistant bacteria. *Advanced Drug Delivery Reviews* **78**:3-13.

Tenaillon, O., D. Skurnik, B. Picard, and E. Denamur. 2010. The population genetics of commensal *Escherichia coli*. *Nature Reviews Microbiology* **8**:207-217.

Tennstedt, T., R. Szczepanowski, S. Braun, A. Puhler, and A. Schluter. 2003. Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant. *FEMS Microbiology Ecology* **45**:239-252.

Ternes, T. A., M. Bonerz, N. Herrmann, B. Teiser, and H. R. Andersen. 2007. Irrigation of treated wastewater in Braunschweig, Germany: an option to remove pharmaceuticals and musk fragrances. *Chemosphere* **66**:894-904.

Thai, P. K., L. X. Ky, V. N. Binh, P. H. Nhung, P. T. Nhan, N. Q. Hieu, N. T. T. Dang, N. K. B. Tam, and N. T. K. Anh. 2018. Occurrence of antibiotic residues and antibiotic-resistant bacteria in effluents of pharmaceutical manufacturers and other sources around Hanoi, Vietnam. *Science of the Total Environment* **645**:393-400.

Thomas, C. P., L. S. Moore, N. Elamin, M. Doumith, J. Zhang, S. Maharjan, M. Warner, C. Perry, J. F. Turton, and C. Johnstone. 2013. Early (2008–2010) hospital outbreak of *Klebsiella pneumoniae* producing OXA-48 carbapenemase in the UK. *International Journal of Antimicrobial Agents* **42**:531-536.

Tóth, K., Á. Tóth, K. Kamotsay, V. Németh, and D. Szabó. 2022. Population snapshot of the extended-spectrum β-lactamase-producing *Escherichia coli* invasive strains isolated from a Hungarian hospital. *Annals of Clinical Microbiology* **21**:1-10.

Touchon, M., J. A. Moura de Sousa, and E. P. Rocha. 2017. Embracing the enemy: the diversification of microbial gene repertoires by phage-mediated horizontal gene transfer. *Current Opinion in Microbiology* **38**:66-73.

Trevors, J. T. 2011. Viable but non-culturable (VBNC) bacteria: Gene expression in planktonic and biofilm cells. *Journal of Microbiology Methods* **86**:266-273.

Tuméo, E., H. Gbaguidi-Haore, I. Patry, X. Bertrand, M. Thouverez, and D. Talon. 2008. Are antibiotic-resistant *Pseudomonas aeruginosa* isolated from hospitalised patients recovered

in the hospital effluents? *International Journal of Hygiene and Environmental Health* **211**:200-204.

Tylová, T., M. Flieger, and J. Olšovská. 2013. Determination of antibiotics in influents and effluents of wastewater-treatment-plants in the Czech Republic–development and application of the SPE and a UHPLC-ToFMS method. *Analytical Methods* **5**:2110-2118.

UN. 2019. No Time to Wait–Securing the Future from Drug-resistant Infections. Report to the Secretary General of the Nations.

Valcarcel, Y., S. G. Alonso, J. L. Rodriguez-Gil, A. Gil, and M. Catala. 2011. Detection of pharmaceutically active compounds in the rivers and tap water of the Madrid Region (Spain) and potential ecotoxicological risk. *Chemosphere* **84**:1336-1348.

Van, T. T. H., Z. Yidana, P. M. Smooker, and P. J. Coloe. 2020. Antibiotic use in food animals worldwide, with a focus on Africa: Pluses and minuses. *Global Antimicrobial Resistance* **20**:170-177.

Vergeynst, L., A. Haeck, P. De Wispelaere, H. Van Langenhove, and K. Demeestere. 2015. Multi-residue analysis of pharmaceuticals in wastewater by liquid chromatography–magnetic sector mass spectrometry: method quality assessment and application in a Belgian case study. *Chemosphere* **119**:S2-S8.

Verlicchi, P., M. Al Aukidy, A. Galletti, M. Petrovic, and D. Barcelo. 2012. Hospital effluent: investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. *Science of the Total Environment* **430**:109-118.

Villegas, M. V., K. Lolans, A. Correa, J. N. Kattan, J. A. Lopez, J. P. Quinn, and G. Colombian Nosocomial Resistance Study. 2007. First identification of *Pseudomonas aeruginosa* isolates producing a KPC-type carbapenem-hydrolyzing beta-lactamase. *Antimicrobial Agents and Chemotherapy* **51**:1553-1555.

Volkmann, H., T. Schwartz, S. Kirchen, C. Stofer, and U. Obst. 2007. Evaluation of inhibition and cross-reaction effects on real-time PCR applied to the total DNA of wastewater samples for the quantification of bacterial antibiotic resistance genes and taxon-specific targets. *Molecular and Cellular Probes* **21**:125-133.

Von Wintersdorff, C. J., J. Penders, J. M. Van Niekerk, N. D. Mills, S. Majumder, L. B. Van Alphen, P. H. Savelkoul, and P. F. Wolffs. 2016. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Frontiers in Microbiology* **7**:173.

Wachino, J.-i., and Y. Arakawa. 2012. Exogenously acquired 16S rRNA methyltransferases found in aminoglycoside-resistant pathogenic Gram-negative bacteria: an update. *Drug Resistance Updates* **15**:133-148.

Walsh, T. R., M. A. Toleman, L. Poirel, and P. Nordmann. 2005. Metallo-β-lactamases: the quiet before the storm? *Clinical Microbiology Reviews* **18**:306-325.

Wang, J., L. Chu, L. Wojnárovits, and E. Takács. 2020. Occurrence and fate of antibiotics, antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) in municipal wastewater treatment plant: an overview. *Science of the Total Environment*:140997.

Wang, J., and S. Wang. 2016. Removal of pharmaceuticals and personal care products (PPCPs) from wastewater: A review. *Journal of Environmental Management* **182**:620-640.

Wang, Q., P. Wang, and Q. Yang. 2018a. Occurrence and diversity of antibiotic resistance in untreated hospital wastewater. *Science of the Total Environment* **621**:990-999.

Wang, X. D., J. C. Cai, H. W. Zhou, R. Zhang, and G.-X. Chen. 2009. Reduced susceptibility to carbapenems in *Klebsiella pneumoniae* clinical isolates associated with plasmid-mediated

β-lactamase production and OmpK36 porin deficiency. *Medical Microbiology* **58**:1196-1202.

Watkinson, A., E. Murby, and S. Costanzo. 2007. Removal of antibiotics in conventional and advanced wastewater treatment: implications for environmental discharge and wastewater recycling. *Water Research* **41**:4164-4176.

Watkinson, A. J., E. J. Murby, D. W. Kolpin, and S. D. Costanzo. 2009. The occurrence of antibiotics in an urban watershed: from wastewater to drinking water. *Science of the Total Environment* **407**:2711-2723.

Wayne, P. 2010. Clinical and Laboratory Standards Institute: Performance standards for antimicrobial susceptibility testing: 20th informational supplement. CLSI document M100-S20.

Weldhagen, G. F., and A. Prinsloo. 2004. Molecular detection of GES-2 extended spectrum Beta-lactamase producing *Pseudomonas aeruginosa* in Pretoria, South Africa. *Intenational Journal of Antimicrobial Agents* **24**:35-38.

White, L., K. Hopkins, D. Meunier, C. L. Perry, R. Pike, P. Wilkinson, R. W. Pickup, J. Cheesbrough, and N. Woodford. 2016. Carbapenemase-producing Enterobacteriaceae in hospital wastewater: a reservoir that may be unrelated to clinical isolates. *Journal of Hospital Infection* **93**:145-151.

WHO. 2019. No Time to Wait–Securing the Future from Drug-resistant Infections. Report to the Secretary General of the Nations.

WHO. 2014. Antimicrobial resistance: global report on surveillance. World Health Organization.

Wiegand, I., H. K. Geiss, D. Mack, E. Stürenburg, and H. Seifert. 2007. Detection of extended-spectrum beta-lactamases among Enterobacteriaceae by use of semiautomated microbiology systems and manual detection procedures. *Clinical Microbiology* **45**:1167-1174.

Woodford, N., J. F. Turton, and D. M. Livermore. 2011. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiology Reviews* **35**:736-755.

Woodford, N., D. W. Wareham, B. Guerra, and C. Teale. 2014. Carbapenemase-producing Enterobacteriaceae and non-Enterobacteriaceae from animals and the environment: an emerging public health risk of our own making? *Journal of Antimicrobial Chemotherapy* **69**:287-291.

Wright, G. D. 2010. Antibiotic resistance in the environment: a link to the clinic? *Current Opinion in Microbiology* **13**:589-594.

Yamane, K., Y. Doi, K. Yokoyama, T. Yagi, H. Kurokawa, N. Shibata, K. Shibayama, H. Kato, and Y. Arakawa. 2004. Genetic environments of the rmtA gene in *Pseudomonas aeruginosa* clinical isolates. *Antimicrobial Agents and Chemotherapy* **48**:2069-2074.

Yamane, K., J.-i. Wachino, S. Suzuki, K. Kimura, N. Shibata, H. Kato, K. Shibayama, T. Konda, and Y. Arakawa. 2007. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. *Antimicrobial Agents and Chemotherapy* **51**:3354-3360.

Yang, C., M. Lin, P. Liao, H. Yeh, B. Chang, T. Tang, C. Cheng, C. Sung, and M. Liou. 2009. Comparison of antimicrobial resistance patterns between clinical and sewage isolates in a regional hospital in Taiwan. *Letters in Applied Microbiology* **48**:560-565.

Yang, S., and K. Carlson. 2004. Solid-phase extraction–high-performance liquid chromatography–ion trap mass spectrometry for analysis of trace concentrations of macrolide antibiotics in natural and waste water matrices. *Chromatography A* **1038**:141-155.

Yang, S., J. Cha, and K. Carlson. 2005. Simultaneous extraction and analysis of 11 tetracycline and sulfonamide antibiotics in influent and effluent domestic wastewater by solid-phase extraction and liquid chromatography-electrospray ionization tandem mass spectrometry. *Chromatography A* **1097**:40-53.

Yu, Y.-s., H. Zhou, Q. Yang, Y.-g. Chen, and L.-j. Li. 2007. Widespread occurrence of aminoglycoside resistance due to ArmA methylase in imipenem-resistant *Acinetobacter baumannii* isolates in China. *Antimicrobial Chemotherapy* **60**:454-455.

Zavascki, A. P., C. G. Carvalhaes, R. C. Picao, and A. C. Gales. 2010. Multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: resistance mechanisms and implications for therapy. *Expert Review of Anti Infective Therapy* **8**:71-93.

Zeng, Z. R., W. P. Wang, M. Huang, L. N. Shi, Y. Wang, and H. F. Shao. 2014. Mechanisms of carbapenem resistance in cephalosporin-susceptible *Pseudomonas aeruginosa* in China. *Diagnostic Microbiology and Infectious Disease* **78**:268-270.

Zhang, T., and B. Li. 2011. Occurrence, transformation, and fate of antibiotics in municipal wastewater treatment plants. *Critical Reviews in EnvironmentalSscience and Technology* **41**:951-998.

Zhang, X., X. Lu, and Z. Zong. 2012. Enterobacteriaceae producing the KPC-2 carbapenemase from hospital sewage. *Diagnostic Microbiology and Infectious Disease* **73**:204-206.

Zhang, X. X., T. Zhang, and H. H. Fang. 2009a. Antibiotic resistance genes in water environment. *Applied Microbiology and Biotechnology* **82**:397-414.

Zhang, X. X., T. Zhang, M. Zhang, H. H. Fang, and S. P. Cheng. 2009b. Characterization and quantification of class 1 integrons and associated gene cassettes in sewage treatment plants. *Applied Microbiology and Biotechnology* **82**:1169-1177.

Zhang, Y., C. F. Marrs, C. Simon, and C. Xi. 2009c. Wastewater treatment contributes to selective increase of antibiotic resistance among *Acinetobacter* spp. *Science of the Total Environment* **407**:3702-3706.

Zhen, X., C. S. Lundborg, X. Sun, X. Hu, and H. Dong. 2019. Economic burden of antibiotic resistance in ESKAPE organisms: a systematic review. *Antimicrobial Resistance and Infection Control* **8**:137.

Zhou, Y., H. Yu, Q. Guo, X. Xu, X. Ye, S. Wu, Y. Guo, and M. Wang. 2010. Distribution of 16S rRNA methylases among different species of Gram-negative bacilli with high-level resistance to aminoglycosides. *European Journal of Clinical Microbiology and Infectious Diseases* **29**:1349-1353.

8.0 LIST OF PUBLICATIONS

MTMT Author ID: 10062135

Articles related to this Thesis

Mutuku, C.; Melegh, S.; Kovacs, K.; Urban, P.; Virág, E.; Heninger, R.; Herczeg, R.; Sonnevend, Á.; Gyenesei, A.; Fekete, C,; Gazdag, Z. Characterization of β-Lactamases and Multidrug Resistance Mechanisms in Enterobacterales from Hospital Effluents and Wastewater Treatment Plant. *Antibiotics* **2022**, 11, 776. https://doi.org/10.3390/ *Antibiotics*11060776 (Q1; IF_{2021}: 5.222) Number of independent citations: 1.

Mutuku, C. Gazdag, Z. Melegh, S. Occurrence of antibiotics and bacterial resistance genes in wastewater: resistance mechanisms and antimicrobial resistance control approaches. *World Journal of Microbiology and Biotechnology* (2022) 38:152 https://doi.org/10.1007/s11274-022-03334-0 (Q2; IF_{2021}: 4.253) Number of independent citations: 5.

Mutuku, C. Kutasy B, Urban P, Melegh S, Herczeg R, Gazdag Z and Virág E. Plasmid sequence dataset of multidrug-resistant Enterobacterales isolated from hospital effluents and wastewater treatment plant. *Applied Microbiology, Data in Brief* (2022) https://doi.org/10.1016/j.dib.2022.108736 (Q4; IF_{2021}:1,38).

Mutuku, **C**. Beta-lactam resistance in Enterobacterales. *Gastroenterology: Medicine and Research* (2022) 7: 1. Paper: 000653, 4p https://*DOI:10.31031/GMR.2022.07.000653* (IF_{2021}: 0.929).

Manuscripts in preparation

Mutuku C, Melegh S, Maász A, Kutasy B, Maász G, Kovács K, Kulágin D, Kálmán N, Virág E, Sonnevend Á, Hadzsiev K, Galambos I, Fekete C, Gazdag Z · Municipal wastewater containing clinical effluents harboring antimicrobial pharmaceuticals disseminates carbapenemase-producing *Pseudomonas aeruginosa.*

Mutuku C, Kovacs K, Melegh S, Meszéna R, Sonnevend A, Fekete C, Gazdag Z. Untreated hospital wastewater discharge disseminates vancomycin resistant enterococci (VRE) into the Environment.

Publications unrelated to this Thesis

Mutuku C, Okemo P and Boga H. 2014. Association of Metal Tolerance with Multidrug Resistance among Environmental Bacteria from Wetlands of Lake Victoria Basin. *Agriculture and Biology Journal of North America (ABJNA)* 5 (1): 24 – 32. doi:10.5251/abjna.2014.5.1.24.32

Mutuku C, Okemo P and Boga H. 2014. Metal pollutants distribution within the Lake Victoria basin and their influence on native and transient microbial flora. *ARPN Journal of Agricultural and Biological Sciences, Jabs* 9(4):127-133

Conference abstracts

Mutuku C., Kovacs K., Melegh S., Heninger R., Kulagin D., Urban Peter., Mestyan Gyula., Sonnevend A., Fekete C., Gazdag Z. (2020). Multiple Antimicrobial Resistance and Molecular Characterization of β-lactamases in enteric bacteria from Hospital Effluents and Wastewater Treatment Plant. Medical Conference for PhD Students and Experts of Clinical Sciences, MEDPECS2020, P.70

Mutuku C., Kovács K., Melegh S., Biro A., Boros V., Heninger R., Urban P., Sonnevend Á., Fekete Csaba., Gazdag Z. (2020). Detection of multidrug resistance and prevalence of extended spectrum β-lactamase (ESBL) producing Enterobacteriaceae from hospital effluents and wastewater treatment plants. XVIII. Szentagothai multidiszciplinaris konferencia es hallgatoi versant JSMC-2020, P.188

Mutuku C., Kovács K., Melegh S., Urban P., Sonnevend Á., Fekete C., Gazdag Z. (2021). Antimicrobial resistance and prevalence of extended spectrum and metallo-β-lactamases in *Pseudomonas aeruginosa* from clinical and municipal wastewater. 18th and 19th János SZENTÁGOTHAI Multidisciplinary Conference and Student Competition, JSMC-2021, P. 26

Mutuku C., Kovács K., Melegh S., Urban P., Sonnevend Á., Fekete C., Gazdag Z. (2021). Detection of Vancomycin Resistant Enterococci (VRE) from Hospital Effluents. Online Medical Conference for PhD Students and Experts of Clinical Sciences, MEDPECS2021, P. 107

11.0 SUPPLEMENTARY MATERIALS

Composition of media applied

Plate Count Agar: For enumeration of live aerobic heterotrophic plate count bacteria. 23.5 g of plate count agar (5.0 tryptone, 2.5 g yeast extract, 1.0 g glucose, 15.0 g agar, and pH 7.2) were dissolved in 1 L of deionized water, heated to dissolve with agitation, and sterilized at 121 ^{0}C for 15 minutes.

Eosin Methylene Blue Agar: For selective isolation of lactose fermenting enteric bacteria. 36 g of Eosin Methylene Blue Agar (10.5 g peptone, 1.0 g, lactose, 0.4 g eosin Y dye, 0.065 g methylene blue, 2.0 g buffer, 13 g agar, and pH 7.0) were dissolved in 1 L of deionized water, heated to dissolve with agitation and sterilized at 121 ^{0}C for 15 minutes. It was amended with 2 µg/ml ceftriaxone or 8 µg/ml imipenem after cooling to a temperature of 45-50 ^{0}C.

Cetrimid Nalidixic Agar: For selective isolation of *Pseudomonas aeruginosa*. 50 g of Cetrimid Nalidixic Agar (25.4 g peptone, 10 g potassium sulfate, 1.4 g magnesium chloride, 0.2 g cetrimid, 0.015 g nalidixic acid, 13 g agar, and pH 7.1) were dissolved in 1 L of deionized water and 10 ml of glycerin added to the mixture as a carbon source. It was heated to dissolve, and sterilized at 121 ^{0}C for 15 minutes. The media was amended with 8 µg/ml ceftazidime or 4 µg/ml gentamycin or 0.5 µg/ml ciprofloxacin after cooling to a temperature of 45-50 ^{0}C.

Brain Heart Infusion Agar: For purification of bacteria cultures. 37 g of Brain Heart Infusion Agar (27.5 g nutrient substrate - brain + heart infusion, peptone, 2.0 g glucose, 5.0 g sodium chloride, 2.5 g buffers, 13.5 g agar, and pH 7.4) were dissolved in 1L of deionized water, heated to dissolve with agitation and sterilized at 121 ^{0}C for 15 minutes.

Mueller Hinton Agar: For antimicrobial susceptibility testing. 38 g of Mueller Hinton Agar (2.0 g, beef extract, 17.5 g, acid hydrolysate of casein, 1.5 g, starch, 17.0 g agar, and pH 7.3) were dissolved in 1 L of deionized water, heated to dissolve, and sterilized at 121 ^{0}C for 15 minutes.

Mueller Hinton Broth: For long term storage of bacterial cultures. 38 g of Mueller Hinton Broth (2.0 g, beef extract, 17.5 g, acid hydrolysate of casein, 1.5 g, and pH 7.3) were dissolved in 1L of deionized water, heated to dissolve, and sterilized at 121 ^{0}C for 15 minutes. 1.5 ml of the broth containing 20% glycerol was mixed with 400 µl of bacterial culture suspension grown overnight in the same broth. The contents were mixed in 2.0 ml Eppendorf tubes and vortexed to disperse

the cells. The tubes were placed at 4 ^{0}C for 2 h for the cellular uptake of glycerol and frozen at −80 ^{0}C. All the media were obtained from Biolab laboratories in Budapest.

Solutions and antibiotic solvents

Phosphate buffered saline (PBS: PH: 7.4): 8 g NaCl, 0.2 g KCl, 1.8 g $Na_2HPO_4.2H_2O$, 0.24 g KH_2PO_4 were dissolved in 1000 ml of deionized water with a final pH of 7.4. The solution was sterilized at 121 ^{0}C for 15 minutes.

Phosphate Buffer (0.01mol/L): 1.79 g $Na_2HPO_4.12H_2O$ and 0.689 g $NaH_2PO_4.H_2O$ were dissolved in 400 ml of distilled water with a final volume of 500 ml and a pH of 7.2. The solution was sterilized at 121 ^{0}C for 15 minutes.

Polysorbates/Tween 80 (0.1%): 1000 µl of Tween 80 was added to 1000 ml of Phosphate buffered saline, pH, 7.4 and the solution was sterilized at 121 ^{0}C for 15 minutes.

Sodium Carbonate Solution: Anhydrous sodium carbonate was prepared at a weight of exactly 10% of ceftazidime antibiotic. It was dissolved in the desired volume of deionized water and the solution was stored at room temperature for up to six hours before use.

Antibiotic stock solutions and working concentrations

Antibiotics added to the bacterial growth media were prepared from the following powdered antibiotics: vancomycin hydrochloride, ceftriaxone sodium salt hemiheptahydrate, imipenem powder, ceftazidime pentahydrate, ciprofloxacin powder, and gentamycin sulfate (Merck, Darmstadt, Germany). Water soluble antibiotics were prepared in sterile deionized water while water insoluble antibiotics were prepared in their recommended solvents and diluents. The antibiotics stock solutions are prepared using the formula (1000/P) X V X C = W, where P= potency of the antibiotic base, V = volume in ml required, C = final concentration of the solution and W = weight of the antimicrobial to be dissolved in V. The antibiotic mixtures were sterilized through sterile syringe filter w/0.45 µm cellulose acetate. The filtrate was stored at −20 ^{0}C until use. From the antibiotic stock solutions, the working concentration was mixed with agar previously cooled to a temperature of 45-50^{0}C before dispensing into Petri dishes. The final concentrations of antibiotics used were: vancomycin 4 µg/ml, ceftriaxone 2 µg/ml, imipenem 8 µg/ml, ciprofloxacin 0.5 µg/ml, gentamicin 4 µg/ml and ceftazidime 8 µg/ml.

Matrix solution for MALDI-TOF MS measurement

10 mg ml^{-1} solution of alpha-cyano-4-hydroxycinnamic acid (HCCA) was solubilized in 1 mL of 50% acetonitrile, 47.5% milli-Q water, and 2.5% trifluoroacetic acid and sonicated for 5 minutes to dissolve the matrix crystals followed by 5 minutes of centrifugation.

Molecular biology solutions

0.5 M Ethylene diaminetetraacetic acid (EDTA): 186.1 g of disodium ethylenediamine tetraacetic acid (EDTA.$2H_2O$) was added to 800 ml of distilled water, pH 8.0, and topped to 1 L.
25X Tris-EDTA (TAE) buffer: 48.4 g Tris base was dissolved in water, and 20 ml 0.5 M EDTA pH 8.0 was added. 11.89 ml of 96% acetic acid was added and topped up to a final volume of 400 ml.
1x Tris-EDTA (TAE) buffer: 40 ml drawn from 25× TAE buffer were mixed with distilled water to a final volume of 1 L.
Lysing solution: 3 % SDS, 50 mM Tris, was prepared by addition of 3 g sodium dodecyl sulfate and 0.605 g Tris into 80 ml distilled water. 20 ml of the solution was adjusted to pH 12.6 with 2 N NaOH and topped up to 25 ml.

10× TBE buffer: 108 g Tris and 55 g boric acid were dissolved in distilled water, 40 ml of 0.5 M EDTA, pH 8.0 added and the solution topped up to 1000 ml.

1× TBE buffer: 100 ml drawn from 10× TBE buffer were mixed with distilled water to a final volume of 1 L.
1.5% agarose electrophoresis gel: 1.5 g agarose powder (quality grade) was added to 100 ml of distilled water and heated to dissolve for 2 min and 30 seconds in a microwave oven.

PCR primer pair stock solutions and working concentration

The stock solution of each pair of oligonucleotides was prepared according to the manufacturer's instructions. The recommended volume of nuclease free water was added to each of the respective oligonucleotide pairs to constitute a final concentration of 100 μM. A 10× dilution was used as the working concentration in each case.

Table S1. Analyzed antibiotic Mass Spectrometer parameters

Compound Name	Abbreviation	CAS Number	Chemical Formula	Monoisotopic mass [m/z]	Precursor ion [m/z]	Qualifier Ion [m/z]
Azithromycin	AZI	29951717	C38H72N2O12	748.51	375,2 (2+)	296
Ciprofloxacin	CIP	85721-33-1	C17H18FN3O3	331.13	332.2	314.1
Clarithromycin	CLA	28928615	C38H69NO13	747.48	748.8	158.2
Erythromycin	ERY	0114-07-08	C37H67NO13	733.46	734.5	83
Norfloxacin	NOR	70458-96-7	C16H18FN3O3	319.13	320.2	302.2
Ofloxacin	OFL	82419-36-1	C18H20FN3O4	361.14	365.2	261.2
Sulfamethoxazole	SMZ	723-46-6	C10H11N3O3S	253.05	254.2	92
Sulfadiazine	SDZ	68-35-9	C10H10N4O2S	250.05	251.1	156
Tetracycline	TET	60-54-8	C22H24N2O8	444.15	445.2	410
Trimethoprim	TRI	738-70-5	C14H18N4O3	290.14	291.2	123.1

Compound Name	Abbreviation	CE [V]	Daughter Ion3 [m/z]	CE [V]	Cone Voltage [V]	t_{ret} [min]	R^2	SPE recovery [%]
Azithromycin	AZI	15	375	15	25	5.9	0.9873	85
Ciprofloxacin	CIP	20	245.2	23	25	4.1	0.9906	17
Clarithromycin	CLA	10	83	30	25	7.9	0.9956	41
Diclofenac	DIC	14	278	8	25	9.1	0.9830	30
Erythromycin	ERY	24	116	25	25	7.6	0.9857	19
Norfloxacin	NOR	24	276.3	17	25	3.9	0.9901	4
Ofloxacin	OFL	17	344	20	25	3.8	0.9636	89
Sulfamethoxazole	SMZ	15	108	24	25	4.1	0.9853	29
Sulfadiazine	SDZ	25			25	1.0	0.9857	23
Tetracycline	TET	12	154	26	25	3.8	0.9836	22
Trimethoprim	TRI	20	261	25	25	3.3	0.9859	52

Table S2. The diversity, number and percentage distribution of the enteric bacteria (n = 126) and *P. aeruginosa* (n = 151) from hospital effluents, nursing home, wastewater treatment stages and urban wastewater samples.

Source	*E. coli*	*K. pneumoniae*	*K. oxytoca*	*E. cloacae*	*Citrobacter* spp	Total No. (%)	*P. aeruginosa No. (%)*
H1	8	10	6	2	1	27 (21)	35 (23)
H2	7	6	6	3	3	25 (20)	21 (14)
H3	4	1	0	1	0	6 (5)	12 (8)
H4	7	7	4	1	3	22 (18)	27 (18)
NH	9	1	0	0	1	11 (8)	11 (7)
INF	6	0	0	1	2	9 (7)	12 (8)
ACSL	9	1	1	1	0	12 (10)	16 (11)
DGSL	2	0	0	0	3	5 (4)	9 (6)
UWW	6	0	0	0	3	9 (7)	8 (5)
Total	**58**	**26**	**17**	**9**	**16**	**126**	**151**

Study sites: H1-H4: hospital effluent, NH: nursing home, INFL: influent. ACSL: activated sludge, DGSL: digested sludge, UWW: urban wastewater, *E. coli, (Escherichia coli), K. pneumoniae, (Klebsiella pneumoniae), K. oxytoca, (Klebsiella oxytoca), E. cloacae, (Enterobacter cloacae),* No. (%), number and percentage of isolates.

Table S3. Sequences, annealing temperatures and product sizes of primer sequences targeting specified β-lactamase encoding genes.

Gene	Sequence (5'-3')	Product size (bp)	Annealing temp (ºC)	Reference
CTX-M-F	TTTGCGATGTGCAGTACCAGTAA	544	51	(Edelstein et al. 2003)
CTX-M-R	CGATATCGTTGGTGGTGCCATA			
SHV-F	ATGCGTTATATTCGCCTGTG	865	49	(Wiegand et al. 2007)
SHV-R	GTTAGCGTTGCCAGTGCTCG			
TEM-F	GCGGAACCCCTATTTG	963	56	(Olesen et al. 2004)
TEM-R	ACCATTGCTTAATCAGTGAG			
IMP-F	GGAATAGAGTGGCTTAAYT	232	52	(Poirel et al. 2011)
IMP-R	TCGGTTTAAYAAAACAACCACC			
KPC-F	CGTCTAGTTCTGCTGTCTTG	798	52	(Poirel et al. 2011)
KPC-R	CTTGTCATCCTTGTTAGGCG			
NDM-F	GGTTTGGCGATCTGGTTTTC	621	52	(Poirel et al. 2011)
NDM-R	CGGAATGGCTCATCACGATC			
OXA-48-F	GCGTGGTTAAGGATGAACAC	438	60	(Poirel et al. 2011)
OXA-48-R	CATCAAGTTCAACCCAACCG			
VIM-F	GATGGTGTTTGGTCGCATA	390	52	(Poirel et al. 2011)
VIM-R	CGAATGCGCAGCACCAG			
SPM-F	AAAATCTGGGTACGCAAACG	271	59	(Sheikh et al. 2014)
SPM-R	CATTATCCGCTGGAACAGG			
VIM primers for PCR amplification				
VIM-F	GGTCTATTTGACCGCGTCTATC	775	50	This study
VIM-R	CTACTCAACGACTGAGCGATTT			
VIM-F	GTTTGGTCGCATATCG	161	43	This study
VIM-R	GGAAGTCCAATTTGCTT			

Table S4. Antimicrobial resistance genes and plasmid replicons detected by next generation sequencing

strain	species	isolation site	antimicrobial susceptibility profile									assembly statistics			cluster
			ceftriaxone	ceftazidime	cefotaxime	cefpodoxime	imipenem	meropenem	trimethoprim/sulfameth.	gentamicin	ciprofloxacin	total length (bp)	number of contigs	N50 (bp)	
EC10	Escherichia coli	Hospital effluent - H3	R	R	R	R	S	S	R	R	R	283,752	58	11,554	G1-a
EC14	Escherichia coli	Hospital effluent - H3	R	R	R	R	S	S	R	S	R	314,119	60	14,064	G1-a
EC19	Escherichia coli	Ativated sludge	R	R	R	R	S	S	R	R	R	308,162	64	11,403	G1-a
EC74	Escherichia coli	Nursing home	R	R	R	R	S	S	R	R	R	279,930	48	13,541	G1-a
EC75	Escherichia coli	Nursing home	R	R	R	R	S	S	R	S	R	241,902	45	24,410	G1-a
EC81	Escherichia coli	Hosptial effluent - H2	R	R	R	R	S	S	R	S	R	245,977	33	25,285	G1-a
EC20	Escherichia coli	Ativated sludge	R	R	R	R	S	S	R	S	R	182,477	33	12,206	G1-b
EC66	Escherichia coli	Hosptial effluent - H4	R	R	R	R	S	S	R	S	R	188,429	19	12,741	G1-b
EC11	Escherichia coli	Hospital effluent - H3	R	R	R	R	S	S	R	R	R	519,759	59	34,711	G1-c
KP2	Klebsiella pneumoniae	Hospital effluent - H1	R	R	R	R	S	S	R	S	R	335,893	27	62,343	G2
KP6	Klebsiella pneumoniae	Hospital effluent - H1	R	R	R	R	S	S	R	R	R	337,552	27	62,343	G2
KP93	Klebsiella pneumoniae	Hospital effluent - H2	R	R	R	R	R	S	R	S	R	317,235	27	58,475	G2
KP96	Klebsiella pneumoniae	Hospital effluent - H2	R	R	R	R	S	S	R	S	R	349,537	35	58,475	G2
KP4	Klebsiella pneumoniae	Influent	R	R	R	R	R	S	S	R	R	276,951	26	47,581	G2
KP45	Klebsiella pneumoniae	Hospital effluent - H2	R	R	R	R	S	S	R	R	R	225,949	24	58,475	G2
KP76	Klebsiella pneumoniae	Hospital effluent - H4	R	R	R	R	S	S	R	S	R	226,186	26	58,475	G2
KP79	Klebsiella pneumoniae	Hospital effluent - H4	R	R	R	R	S	S	R	S	R	226,283	24	19,616	G2
CF102	Citrobacter freundii	Digested sludge	R	R	R	R	S	S	R	R	R	547,810	48	28,183	G3
KP57	Klebsiella pneumoniae	Hospital effluent - H4	R	R	R	R	R	R	S	S	R	467,511	37	29,555	G4
KO54	Klebsiella oxytoca	Hospital effluent - H4	R	R	R	R	R	S	S	R	R	459,323	62	30,268	G5
EC92	Escherichia coli	Hosptial effluent - H1	R	R	R	R	S	S	S	S	R	190,351	17	16,365	G6

Table S4. cont'd

extended spectrum β-lactamase genes					other β-lactamase genes			aminoglycoside resistance genes							amphenicol resistance genes			folate inhibitor resistance genes				other resistance genes			
bla$_{CTX-M-1}$	*bla*$_{CTX-M-15}$	*bla*$_{CTX-M-27}$	*bla*$_{CTX-M-30}$	*bla*$_{SHV-12}$	*bla*$_{TEM-1}$	*bla*$_{OXA-1}$	*bla*$_{OXA-9}$	*aac(3)-IIa*	*aac(6')-Ib*	*aac(6')-Ib-cr**	*aadA1*	*aadA5*	*aph(3'')-Ib*	*aph(6)-Id*	*catB3*	*cmlB1*	*floR*	*dfrA14*	*dfrA17*	*sul1*	*sul2*	*mph(A)*	*tet(A)*	*qacE*	*qnrB1*
		X			X							X	X	X			X		X	X	X	X	X	X	
		X			X							X	X	X			X		X	X	X	X	X	X	
		X			X							X	X	X			X		X	X	X	X	X	X	
		X			X							X	X	X			X		X	X	X	X	X	X	
		X			X							X	X	X			X		X	X	X	X	X	X	
		X			X							X	X	X			X		X	X	X	X	X	X	
		X										X	X	X					X	X	X	X	X	X	
		X										X	X	X					X	X	X	X	X	X	
		X										X	X	X					X	X	X	X	X	X	
	X				X	X				X			X	X	X			X			X				X
	X				X	X				X			X	X	X			X			X				X
	X				X	X		X		X			X	X	X			X			X				X
	X				X	X		X		X			X	X	X			X			X				X
	X				X	X		X		X			X	X	X			X			X				X
	X				X	X				X			X	X	X			X			X				X
	X				X	X				X			X	X	X			X			X				X
	X					X		X		X			X		X	X							X		
				X	X		X		X		X		X	X											
			X		**		X		X		X														
X																									

Table S4. Cont'd

plasmid replicons											
IncA	IncB/O/K/Z	IncC	IncFIA	IncFIB	IncFII	IncI	IncN	IncN2		IncX1	other
	X		X	X	X						
	X		X	X	X						
	X		X	X	X						
	X		X	X	X						
	X		X	X	X						
	X		X	X	X						
			X		X	X					
			X		X	X					
			X	X	X	X					
				X	X						
				X	X						
				X	X						
				X	X						
				X	X						
				X	X						
				X	X						
				X	X						
X					X		X				
		X			X			X			Col(IMGS31), pKPC-CAV1321
			X		X				X		Col440I
				X	X					X	

Table S5. Primer sequences used for the VIM gene sequencing, their annealing temperature and the amplicon length.

Variant	Primer sequences	Annealing temperature (°C)	Amplicon length (bp)
rs662799	F: 5'-CCCCAGGAACTGGAGCGACCTT-3'	55	398
	R 5'-TTCAAGCAGAGGGAAGCCTGTA-3'		
rs2266788	F: 5'-TCAGTCCTTGAAAGTGGCCT-3'	64	287
	R: 5'-ATGTAGTGGCACAGGCTTCC-3'		
rs3135506	F: 5'-AGAGCTAGCACCGCTCCTTT-3'	64	256
	R: 5'-TAGTCCCTCTCCACAGCGTT-3'		
rs2072560	F: 5'-CTCAAGGCTGTCTTCAG-3'	62	280
	R: 5'-CCTTTGATTCTGGGGACTGG-3'		

Table S6 (a). Samples of MALDI-TOF/MS run data of enteric bacteria and *P. aeruginosa.*

Result overview table--continued from previous page

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
A8 (+++) (C)	6 (standard)	Klebsiella oxytoca	2.23	Raoultella ornithinolytica	2.21
A9 (+++) (C)	6 (standard)	Pseudomonas aeruginosa	2.37	Pseudomonas aeruginosa	2.32
A10 (+++) (A)	7 (standard)	Pseudomonas aeruginosa	2.45	Pseudomonas aeruginosa	2.26
A11 (+++) (A)	8 (standard)	Pseudomonas aeruginosa	2.51	Pseudomonas aeruginosa	2.41
A12 (+++) (A)	9 (standard)	Pseudomonas aeruginosa	2.38	Pseudomonas aeruginosa	2.30
B1 (+++) (A)	10 (standard)	Klebsiella pneumoniae	2.31	Klebsiella pneumoniae	2.23
B2 (+++) (A)	10 (standard)	Klebsiella pneumoniae	2.25	Klebsiella pneumoniae	2.24
B3 (+++) (A)	11 (standard)	Klebsiella pneumoniae	2.33	Klebsiella pneumoniae	2.32
B4 (+++) (A)	12 (standard)	Pseudomonas aeruginosa	2.30	Pseudomonas aeruginosa	2.29
B5 (+++) (C)	13 (standard)	Klebsiella pneumoniae	2.41	Klebsiella pneumoniae	2.33
B6 (+++) (C)	13 (standard)	Klebsiella pneumoniae	2.26	Klebsiella pneumoniae	2.20
B7 (+++) (C)	13 (standard)	Pseudomonas aeruginosa	2.34	Pseudomonas aeruginosa	2.34
B8 (+++) (A)	14 (standard)	Pseudomonas aeruginosa	2.45	Pseudomonas aeruginosa	2.41
B9 (+++) (A)	15 (standard)	Pseudomonas aeruginosa	2.43	Pseudomonas aeruginosa	2.43
B10 (+++) (A)	16 (standard)	Pseudomonas aeruginosa	2.33	Pseudomonas aeruginosa	2.33
B11 (+++) (A)	17 (standard)	Pseudomonas aeruginosa	2.44	Pseudomonas aeruginosa	2.32

Result overview table--continued on next page

Table S6 (b). Detailed examination data of the A10 *P. aeruginosa* sample from the Maldi database.

Sample 10

Sample Name:	A10
Sample Description:	
Sample ID:	7
Sample Creation Date/Time:	2019-06-28T12:27:51.259
Sample Type:	Standard
Identification Method:	MALDI Biotyper MSP Identification Standard Method 1.1
Preprocessing Method:	MALDI Biotyper Preprocessing Standard Method 1.1
ACQ Method:	D:\Methods\flexControlMethods\MBT_FC.par
ACQ Timestamp:	2019-06-28T12:29:59.095
AutoXecute Method:	MBT_AutoX_smart_2
Applied MSP Library(ies):	BDAL / contains 7311 MSPs / 411eaddf-ac6a-4648-afdc-73fcdd8d7777 / 2017-08-21T17:19:16.779, Mycobacteria Library (bead method) / contains 912 MSPs / 8499d296-74af-4783-b40d-53a997f865a5 / 2017-08-22T17:57:09.363, Filamentous Fungi / contains 364 MSPs / 5887a341-a4ad-4a22-8aac-da2ccfa96cb9 / 2017-08-22T19:34:35.083

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (-+++)	Pseudomonas aeruginosa ATCC 27853 THL	2.45	287
2 (-+++)	Pseudomonas aeruginosa DSM 50071T_QC DSM	2.26	287
3 (-+++)	Pseudomonas aeruginosa DSM 50071T HAM	2.24	287
4 (-+++)	Pseudomonas aeruginosa 19955_1 CHB	2.23	287
5 (-+++)	Pseudomonas aeruginosa DSM 1117 DSM	2.22	287
6 (-+++)	Pseudomonas aeruginosa S147_2 CHB	2.21	287
7 (-+++)	Pseudomonas aeruginosa LMG 8029 LMG	2.16	287
8 (-+++)	Pseudomonas aeruginosa A07_08_Pudu FLR	2.08	287
9 (-+++)	Pseudomonas aeruginosa DSM 1128 DSM	2.00	287

Result table for sample 10--continued on next page

Bruker MALDI Biotyper Identification Results

Run Info:

Run Identifier:	191129-1305-1011011985
Comment:	
Operator:	tof-user
Run Creation Date/Time:	2019-11-29T13:12:02.438
Number of Tests:	96
Type:	Standard
BTS-QC:	not present
BTS-QC Position:	
Instrument ID:	8269944.03146
Server Version:	4.1.70 (PYTH) 48 2016-10-26_15-05-35

Result Overview

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
A1 (+) (B)	1 (standard)	Stenotrophomonas maltophilia	1.95	Stenotrophomonas maltophilia	1.93
A2 (+++) (A)	2 (standard)	Klebsiella pneumoniae	2.30	Klebsiella pneumoniae	2.29
A3 (+++) (C)	3 (standard)	Stenotrophomonas maltophilia	2.23	Stenotrophomonas maltophilia	2.13
A4 (+++) (C)	3 (standard)	Ochrobactrum anthropi	2.44	Ochrobactrum anthropi	2.43
A5 (+++) (A)	4 (standard)	Escherichia coli	2.41	Escherichia coli	2.39
A6 (+++) (A)	5 (standard)	Aeromonas hydrophila	2.26	Aeromonas hydrophila	2.15
A7 (+++) (A)	6 (standard)	Aeromonas hydrophila	2.34	Aeromonas hydrophila	2.34

Result overview table--continued on next page

Table S6 (c). Detailed examination data of the A5 *E. coli* sample from the Maldi database.

Sample 5

Sample Name:	A5
Sample Description:	
Sample ID:	4
Sample Creation Date/Time:	2019-11-29T13:12:02.463
Sample Type:	Standard
Identification Method:	MALDI Biotyper MSP Identification Standard Method 1.1
Preprocessing Method:	MALDI Biotyper Preprocessing Standard Method 1.1
ACQ Method:	D:\Methods\flexControlMethods\MBT_FC.par
ACQ Timestamp:	2019-11-29T13:13:15.850
AutoXecute Method:	MBT_AutoX_smart
Applied MSP Library(ies):	BDAL / contains 7311 MSPs / 411eaddf-ac6a-4648-afdc-73fcdd8d7777 / 2017-08-21T17:19:16.779, Mycobacteria Library (bead method) / contains 912 MSPs / 8499d296-74af-4783-b40d-53a997f865a5 / 2017-08-22T17:57:09.363, Filamentous Fungi / contains 364 MSPs / 5887e341-a4ad-4a22-8aac-de2ccfa96cb9 / 2017-08-22T19:34:35.083

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (+++)	Escherichia coli RV412_A1_2010_06a LBK	2.41	562
2 (+++)	Escherichia coli DSM 1576 DSM	2.39	562
3 (+++)	Escherichia coli MB11464_1 CHB	2.27	562
4 (+++)	Escherichia coli B421 UFL	2.26	562
5 (+++)	Escherichia coli DSM 682 DSM	2.26	562
6 (+++)	Escherichia coli Nissl VML	2.22	562
7 (+++)	Escherichia coli ATCC 35218 CHB	2.16	562
8 (+++)	Escherichia coli ATCC 25922 CHB	2.11	562
9 (+++)	Escherichia coli DSM 1103_QC DSM	2.08	562

Result table for sample 5--continued on next page

Meaning of Score Values

Range	Interpretation	Symbols	Color
2.00 - 3.00	High-confidence identification	(+++)	green
1.70 - 1.99	Low-confidence identification	(-)	yellow
0.00 - 1.69	No Organism Identification Possible	(-)	red

Meaning of Consistency Categories (A - C)

Category	Interpretation
(A)	**High consistency:** The best match is a high-confidence identification. The second-best match is (1) a high-confidence identification in which the species is identical to the best match, (2) a low-confidence identification in which the species or genus is identical to the best match, or (3) a non-identification.
(B)	**Low consistency:** The requirements for high consistency are not met. The best match is a high- or low-confidence identification. The second-best match is (1) a high- or low-confidence identification in which the genus is identical to the best match or (2) a non-identification.
(C)	**No consistency:** The requirements for high or low consistency are not met.

Reference gene >AY135661.1 *Pseudomonas aeruginosa* class 1 integron metallo-beta-lactamase (*blaVIM-4*) gene, complete cds whose sequence similarity was compared to the VIM gene from *P. aeruginosa* isolates

CGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTATGCCGCACCCACCCCTATGGA
GTCTTGATGTTAAAAGTTATTAGTAGTTTATTGGTCTACATGACCGCGTCTGTCATGGCTGTC
GCAAGTCCGTTAGCCCATTCCGGGGAGCCGAGTGGTGAGTATCCGACAGTCAACGAAATTCC
GGTCGGAGAGGTCCGACTTTACCAGATTGCCGATGGTGTTTGGTCGCATATCGCAACGCAGT
CGTTTGATGGCGCGGTCTACCCGTCCAATGGTCTCATTGTCCGTGATGGTGATGAGTTGCTTT
TGATTGATACAGCGTGGGGTGCGAAAAACACAGCGGCACTTCTCGCGGAGATTGAAAAGCA
AATTGGACTTCCCGTAACGCGTGCAGTCTCCACGCACTTTCATGACGACCGCGTCGGCGGCG
TTGATGTCCTTCGGGCGGCTGGGGTGGCAACGTACGCATCACCGTCGACACGCCGGCTAGCC
GAGGCAGAGGGGAACGAGATTCCCACGCATTCTCTAGAAGGACTCTCATCGAGCGGGGACG
CAGTGCGCTTCGGTCCAGTAGAGCTCTTCTATCCTGGTGCTGCGCATTCGACCGACAATCTG
GTTGTATACGTCCCGTCAGCGAACGTGCTATACGGTGGTTGTGCCGTTCATGAGTTGTCACG
CACGTCTGCGGGGAACGTGGCCGATGCCGATCTGGCTGAATGGCCCACCTCCGTTGAGCGGA
TTCAAAAACACTACCCGGAAGCAGAGGTCGTCATTCCCGGGCACGGTCTACCGGGCGGTCTA
GACTTGCTCCAGCACACAGCGAACGTTGTCAAAGCACACAAAAATCGCTCAGTCGCCGAGT
AGCA