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PhD THESIS

Molecular Epidemiology and Antimicrobial Resistance Spread of Environmental *Escherichia coli* Isolates «A One Health Approach»

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PhD THESIS

Molecular Epidemiology and Antimicrobial Resistance Spread of Environmental *Escherichia coli* Isolates

«A One Health Approach»

Chrysoula E. Dioli

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Author's Declaration for Doctoral Thesis

I, the undersigned, Chryssoula Dioli, PhD candidate in the Department of Biomedical Sciences, School of Health and Care Sciences, University of West Attica, hereby declare that:

I am the author and holder of the intellectual property rights to this thesis and have not infringed upon the intellectual property rights of others. In the preparation of my doctoral thesis, I did not use any part or whole of another creator's work, nor the ideas or opinions of another creator, without proper citation of the source (*book, article from a newspaper or journal, website, etc.*).

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DISSEMINATION OF RESULTS - PUBLICATION

During the course of my doctoral research, the findings from both my dissertation and the additional projects I participated in were published in internationally peer-reviewed scientific journals and presented at national and international conferences.

Publications in international scientific journals

Dioli Chrysoula, Pappa Olga, Siatravani Eirini, Bratakou Spiridoula, Tatsiopoulos Apostolos, Giakkoupi Panagiota, Miriagou Vivi & Beloukas Apostolos (2023). Molecular Characterization and Prevalence of Antimicrobial-Resistant *Escherichia coli* Isolates Derived from Clinical Specimens and Environmental Habitats, *microorganisms*, 11 (6), 1399
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Olga Pappa, Anastasia-Maria Kefala, Kyriaki Tryfinopoulou, Marios Dimitriou, Kostas Kostoulas, Chrysoula Dioli, Eleni Moraitou, Maria Panopoulou, Evaggelos Vogiatzakis, Athena Mavridou, Alexis Galanis, Apostolos Beloukas (2020). Molecular Epidemiology of Multi-Drug Resistant Pseudomonas aeruginosa Isolates from Hospitalized Patients in Greece. microorganisms, 8 (11), 1652.
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Olga Pappa, Dimosthenis Chochlakis, Vassilios Sandalakis, Chrysoula Dioli, Anna Psaroulaki, and Athena Mavridou (2020). Antibiotic Resistance of Legionella pneumophila in Clinical and Water Isolates—A Systematic Review, *Int. J. Environ. Res. Public Health*, 17 (16), 5809;
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Conferences

1. Participation in the 1st Conference of the Hellenic Scientific Society of Aesthetics, as a speaker in the Round Table titled "What an Aesthetic Professional Should Know," with the presentation topic: "Analysis of the Microbiome of Recreational Waters," Athens, Aigaleo, December 2, 2023.

2. Participation in the 13th Panhellenic Conference of Panhellenic Union of Bioscientists, "A New Era for Biology: Opportunities and Challenges in the Post-COVID Era," Thessaloniki, December 9-12, 2022, where the presentation titled "Study of Resistant Environmental and Clinical Strains of *Escherichia coli*" was given as an oral presentation.

3. Participation in the 13th Panhellenic Public Health Conference 2022, "Eudaimonia: The Eternal Goal of Public Health," Athens, February 28 - March 2, 2022, where the presentation titled "Study of Resistance Profiles of *E. coli* Strains Isolated from Dairy Farms" was given as an oral presentation.

4. Participation in the 12th Panhellenic Conference "Bioscientists and One Health: Humans, Animals, Environment," Athens, November 27-29, 2020, where the study titled "Study of Resistance Profiles of Environmental *Escherichia coli* Strains from Livadeia" was presented as a poster.

5. Participation in the 6th Panhellenic Conference of Medical Laboratory Technologists titled "Biomedical Practices and Technological Challenges in Clinical and Research Laboratories," Athens, March 29-31, 2019, where the presentation titled "Study of Resistance Profiles of *E. coli* Strains Isolated from Two Central Swimming Pools in Athens" was given as an oral presentation.

6. Participation in the 8th International Conference on Swimming Pools and Spa, Marseille, France, March 18-22, 2019, where the presentation titled "Resistance Profiles of *E. coli* Isolates Deriving from Two Central Swimming Pools of Athens, Greece" was given as an oral presentation.

Table of Contents

List of Figures

List of Tables

Abstract

Antibiotic resistant bacteria are present in wastewaters as their elimination during treatment in wastewater treatment plants is often impossible. Water plays an important role in the spread of these microorganisms among humans, animals and the environment. Unfortunately, in Greece knowledge on prevalence and diversity of antibiotic resistance bacteria in environmental habitats is very limited.

Therefore, this doctoral dissertation was designed to study antimicrobial resistance under the One Health approach and aimed to a) assess the antibiotic resistance patterns and detect the antibiotic resistance genes related to resistant phenotypes, b) identify molecular genotypes, c) compare resistance patterns and genotypes between clinical and environmental *E. coli* isolates and d) identify molecular mechanism contributing to antimicrobial resistance spread both in clinical settings and in environment (aquatic and wastewater). For this reason, during the thesis, a total of 139 clinical and 502 environmental *E. coli* isolates were collected. Environmental isolates were obtained from semi-treated hospital wastewater, treated wastewater, and river water samples. All these isolates (clinical and environmental) are spatially and temporally related. In order to examine the circulated phylogenies in the clinical settings and in different environmental habitats all isolates were subjected to the molecular typing technique of phylogrouping. This method shown that the phylogenetic group B2 was predominant in clinical settings (60%; 84/139) and the second most frequent among wastewaters, whereas group A was dominant in all environmental isolates (48%, 242/502). To determine the prevalent resistance patterns, all isolates (both clinical and environmental) were evaluated for their susceptibility to 18 commonly used antibiotics. Based on the results, the vast majority of both environmental and clinical isolates were resistant, particularly to penicillins. In addition, 84 isolates (73 environmental and 11 clinical) exhibiting resistant or multidrug-resistant profiles associated with β-lactamases were identified and analyzed for β-lactamase genes. The blaCTX-M-group 1 gene was found in 52 isolates (62%; 52/84), making it the most frequently encountered β-lactamase gene among both clinical and environmental isolates. Other β-lactamase genes detected included blaCTX-M-group 9 (8.4%; 7/84), blaTEM (14.3%; 12/84), blaSHV (20.2%; 17/84), blaOXA-244 (1.2%; 1/84), blaCMY-2 (2.4%; 2/84), blaDHA-1 (1.2%; 1/84), and blaFOX-17 (1.2%; 1/84). Finally, plasmid analysis, conjugation assay and plasmid sequencing were implemented in certain β- lactamase producing isolates to investigate the molecular environment of resistance genes and others molecular mechanisms which probable contributing to resistance dissemination. Out of the 33 isolates initially selected for the conjugation assay, only thirteen (39.4%; 13/33) appeared to contain conjugative plasmids and consequently the ability to transmit resistance το β-lactamases. Sequencing analysis was applied in three plasmids which were isolated from one clinical and two environmental *E. coli* and carried β-lactamase genes. Specifically, the three plasmids were ptrc203cli, ptrc618, and ptrc297, which respectively carried the β-lactamase genes blaDHA-1, blaCTX-M-14, and blaSHV-12. The first two plasmids belong to the compatibility group IncFII, while the last one belongs to the IncX3 group. Additionally, these conjugative plasmids not only carried the aforementioned β-lactamase genes but also additional resistance genes related to resistance to other categories of antibiotics. Specifically, ptrc203cli also co- carried resistance genes for sulfonamides (sul1), trimethoprim (drfA17), and fluoroquinolones (qnrB4); the plasmid ptrc618 harbored resistance genes for aminoglycosides (aac6'-Ib3), macrolides (mphA), and chloramphenicol (cmlA1); and ptrc297 carried a resistance gene for quinolones (qnrS1). The results also showed that all of the resistance genes were embedded within mobile elements (IS elements and integrons), which contribute to the further spread of multidrug resistance.

In conclusion, this doctoral thesis reports confirming data that river water and wastewater serve as reservoirs of antibiotic resistant bacteria and as vehicles for the transmission of resistance genes to various bacterial species.

Keywords: ***E. coli;*** **environment; antibiotic resistance**

Chapter 1

Theoretical Part

Introduction

The rise of antimicrobial resistance (AMR) to nearly all clinically significant antibiotics represents an urgent global health threat that could undermine a century of medical advancements [**1, 2**]. AMR reduces the effectiveness of antimicrobials, resulting in increased morbidity and mortality rates [**1, 2**]. Antibiotic-resistant bacteria (ARB) are not limited to clinical environments but are disseminated through various ecological pathways [**3, 4, 5, 6, 7**]. This phenomenon is largely driven by the selective pressure exerted by the use of antimicrobials in human and veterinary medicine, agriculture, and aquaculture [**8, 9, 10**]. Substantial quantities of antimicrobial residues are released into the environment through several channels, including effluents from wastewater treatment plants (WWTPs), disrupting the equilibrium between sensitive and resistant bacterial populations [**11, 12, 13, 14**].

WWTPs process large volumes of municipal and industrial waste daily, including hospital wastewater (HWW), which contains ARB and antibiotic resistance genes (ARGs) [**1, 14, 15, 16**]. Both ARB and ARGs can evade treatment, and WWTP effluents (a) provide conducive conditions for ARB proliferation and horizontal gene transfer of ARGs, (b) are frequently discharged into aquatic environments such as rivers, seas, and lakes, and (c) are reclaimed for industrial or irrigation purposes in many countries, thereby facilitating the further dissemination of AMR in the ecosystem [**3, 17, 18**]. Systematic monitoring of wastewater is critical for detecting the presence and release of ARB into the environment, which is essential for the safe reuse of treated wastewater [**2, 7**]. Additionally, wastewater surveillance offers insights into the ARB and ARGs circulating within the community.

Although the resistance of *E. coli* to last-resort antibiotics, commonly used in clinical settings, livestock farming, and aquaculture, has been extensively studied in hospital settings, there is limited data available on its presence in community and environmental contexts. *E. coli* is capable of causing serious infections in both humans and animals and is also a member of the indigenous microbiota. Furthermore, *E. coli* serves as a significant reservoir of resistance genes, which can lead to therapeutic failures in human medicine. Numerous resistance genes have been identified in *E. coli*, many of which are transferable through horizontal gene transfer [**3, 4, 6**]. *E. coli* can function as both a donor and recipient of resistance genes. The transmission of virulent and resistant *E. coli* strains between aquatic environments and humans is a major concern, and this can occur through direct contact or via the food chain. Therefore, the genetic background of resistance genes and the circulating phylogenetic groups of antibiotic-resistant *E. coli* isolates in environmental settings are of increasing importance and warrant close monitoring and investigation [**3, 4, 6, 19**]. The following section delves into the fundamental properties and classifications of antibiotic agents.

1.1 Antibiotic agents

Antibiotics (or antibacterials) are chemical substances that kill or inhibit the growth of bacteria without harming the host. They are used to prevent or treat infections caused by bacteria in humans, animals and plants. Antibiotics are produced in nature by fungi, actinomycetes and bacteria, which contribute to the diversity of natural antibiotic compounds. The antibacterial agents derived from natural sources (e.g. benzylpenicillin and gentamicin) are usually chemically modified to improve their antibacterial or pharmacological properties and referred as semi-synthetic (ampicillin and amikacin). Some other agents are totally synthetic (e.g. moxifloxacin and norfloxacin) [**20**].

Antibacterial agents can be classified based on various criteria:

- **Mechanism of action: bactericidal vs. bacteriostatic**: Bactericidal agents eliminate bacteria, whereas bacteriostatic agents merely inhibit bacterial growth. Therefore, bactericidal action results in irreversible bacterial death, while bacteriostasis is a reversible process [**20**].
- **Target site of action**: Antibacterial agents can be categorized based on their primary target within bacterial cells. The five main targets are (a) cell wall synthesis, (b) protein synthesis, (c) nucleic acid synthesis, (d) metabolic pathways, and (e) cell membrane function [**21**] (**Table 1.1, Figure 1.1**).
- **Spectrum of activity**: Antibiotics are classified as broad-spectrum or narrow-spectrum. Broad-spectrum antibiotics are effective against a wide range of bacteria, including both Gram-positive and Gram-negative species, whereas narrow-spectrum antibiotics target either Gram-positive or Gram-negative bacteria specifically [**20**].
- **Chemical structure**: Antibiotics are also grouped by their chemical structure, which is directly linked to their unique therapeutic properties. Based on this criterion, antibiotics are classified into several categories, including β-lactams, macrolides, tetracyclines, aminoglycosides, quinolones, glycopeptides, macrolides, and miscellaneous agents (e.g., sulfonamide-trimethoprim) [**20**].

<table>
<tr><th colspan="3">Table 1.1 Antimicrobial groups based on mechanism of action [20, 21].</th></tr>
<tr><th colspan="2">Mechanism of Action</th><th>Antimicrobial Groups</th></tr>
<tr><td colspan="2">Cell Wall Synthesis Inhibition</td><td>β-Lactams:
• Carbapenems
• Cephalosporins
• Monobactams
• Penicillins
Glycopeptides</td></tr>
<tr><td colspan="2">Cell Membrane Depolarization</td><td>Lipopeptides</td></tr>
<tr><td colspan="2">Metabolic Pathways Inhibition</td><td>Sulfonamides
Trimethoprim</td></tr>
<tr><td rowspan="2">Protein Synthesis Inhibition</td><td>Bind to 30S Ribosomal Subunit</td><td>Aminoglycosides
Tetracyclines</td></tr>
<tr><td>Bind to 50S Ribosomal Subunit</td><td>Chloramphenicol
Lincosamides
Macrolides
Oxazolidinones
Streptogramins</td></tr>
<tr><td colspan="2">Nucleic Acid Synthesis Inhibition</td><td>Quinolones</td></tr>
<tr><td colspan="2">Outer Membrane Disruption</td><td>Colistin (Polymyxin E)</td></tr>
</table>

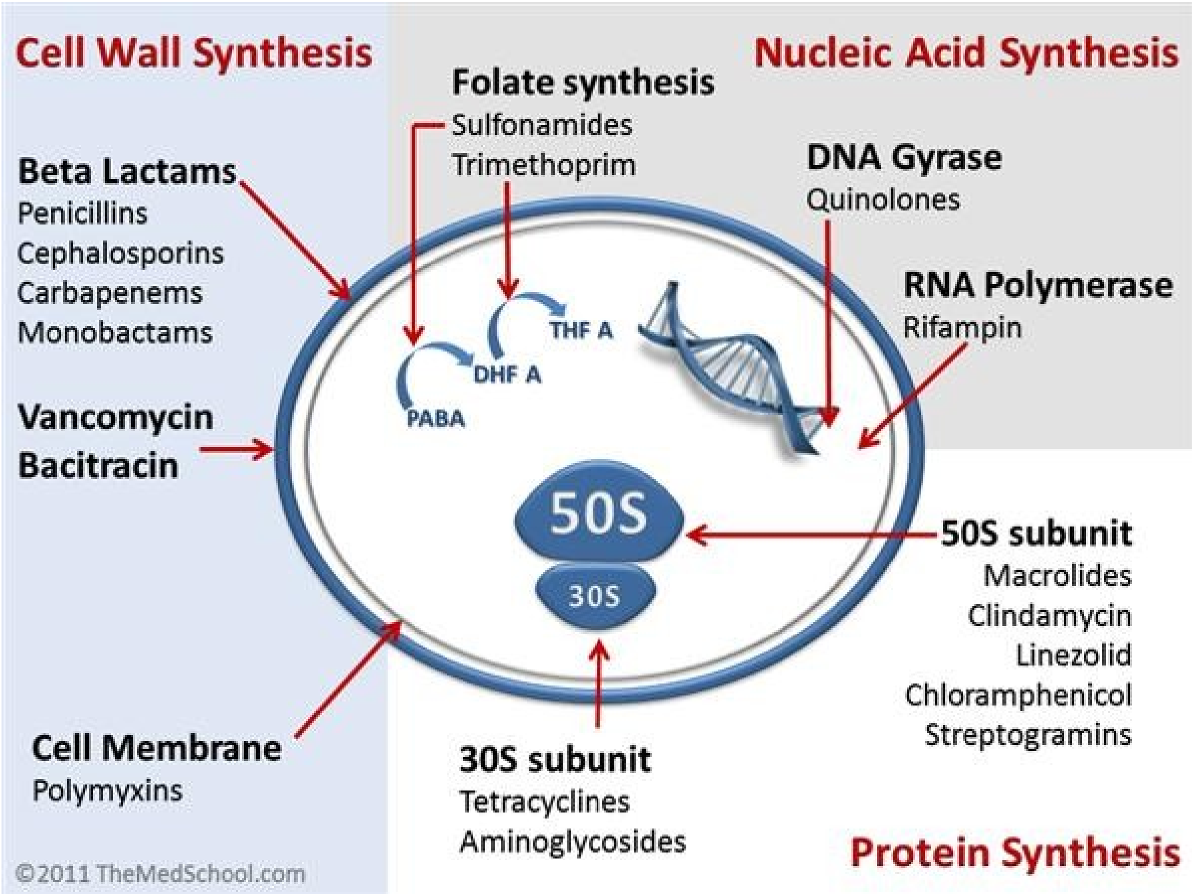


Figure 1.1 Target sites of different antibiotic categories [22]

1.2 Antimicrobial resistance (AMR): definition

Antimicrobial resistance (AMR) is the ability of a microorganism to survive and resist exposure to antimicrobial drug. In the medical setting, the term 'resistant microorganism' is defined as one that will not be inhibited or killed by an antibacterial agent at concentrations of the drug achievable in the body after normal dosage [**2, 20, 21, 23**].

Before discussing the various aspects of antimicrobial resistance, the distinction between natural and acquired resistance should be mentioned. Not all antibiotics are active against all bacterial species. Some species have endogenous/intrinsic resistance to certain antibiotic categories [**20, 21, 23]**. These intrinsic resistance traits are known and predetermined. In other cases, some bacterial strains become resistant to antibiotics in their spectrum. This type of resistance call acquired and it is what the public health is concerned about [**20, 21, 23**].

1.3 Factors contributing to the AMR spread

AMR is a natural phenomenon that is primarily triggered by the selective pressure of antimicrobial use in human and veterinary medicine, agriculture and aquaculture [**8, 9, 10**]. Constantly, significant amounts of antimicrobial residues are released into the environment, and in particular into the aquatic environment, via various routes. As a result, susceptible bacteria are killed, while bacteria that are intrinsically resistant or that have acquired antibiotic-resistant traits have a greater chance to survive and multiply **[11, 12, 13, 14**]. Furthermore, under unfavorable conditions, such as high antibiotic concentration, microorganisms possessing defense strategies endure and proliferate [**23, 24, 25, 26**]. Their strategies for protecting against antibiotics are called resistance mechanisms and are briefly described in **Table 1.2**. **Figure 1.2** illustrates the main mechanisms of bacterial resistance to antibiotics [**23**]. Advantageous chromosomal mutations or exogenous genetic elements acquisition can lead to antibiotic tolerance [**20-26**]. These resistance traits can be inherited generation to generation (vertical transfer) as well as pass directly from bacterium to bacterium (horizontal gene transfer, HGT) via conjugation, transduction, or transformation mode (**Figure 1.3**) [**20-26, 28**]. Horizontal gene transfer (HGT) and mobile genetic elements (MGEs) play a crucial role in the spread of antibiotic resistance genes (ARGs) within and between species [**20-26, 28**]. MGEs such as plasmids, transposons, insertion sequences and integrons contribute to the dissemination of various ARGs due to their ability to move from one location to another within the cell or be transferred from cell to cell horizontally [**28**]. Very often, MGEs harbor multiple resistance genes that confer a multidrug resistance (MDR) phenotype to their hosts [**20-26, 28**].

For the reasons above, the microbiota and microbiome of many environmental habitats have undergone excessive changes due to the increase of ARB and the subsequent accumulation of ARGs, which are present in both extracellular and intracellular forms in the environment. In these settings antibiotics, ARBs, ARGs, and the environmental bacterial flora can interact **[21-28**].

Soil, aquatic environments and wastewaters are identified as reservoir of ARB and ARGs and as ideal settings for development of new ARB via horizontal ARGs tranfer. Specifically, water and wastewaters are regarding a major ways of dissemination of ARB between different environmental compartments [**21-28**].

Wastewater treatment plants (WWTPs) receive vast quantities of municipal and industrial waste daily, including hospital wastewater (HWW) that contains ARB and ARGs [**1, 14, 15, 16**]. ARB and ARGs can evade treatment, and WWTP effluents (a) are often discharged into water bodies such as rivers, seas and lakes and (b) are reclaimed for industrial or irrigation purposes, in many countries, thus contributing to the further spread of AMR in the ecosystem [**3, 17, 18**].

Table 1.2 The main mechanisms of antimicrobial resistance [24]	
Antibiotic Resistance Mechanism	**Description**
Restrict assess of an antimicrobial agent due to changes in membrane permeability	The LPS layer in gram-negative bacteria provides innate resistance to certain antimicrobial groups of antimicrobials Porin-mediated antibiotic resistance: Porins are transmembrane proteins which form channels and normally exists found within the outer membrane of gram-negative bacteria. The porin channel allows the exchange of nutrients and other substances (including antibiotics such as β-lactams and fluoquinolones) between the extracellular environments. The loss or reduced number of porins present in the outer membrane or mutations that change the selectivity of the porin channel prevents the absorption of external substances such as antibacterial compounds [**25**]
Rapid efflux of antimicrobial agents due to over-expression of efflux pumps	Efflux pumps are proteins that are imbedded in the cytoplasmatic membrane of the bacterium. The primary efflux pump function is to remove harmful substances from a bacterial cell. Many of these pumps will transport a large variety of compounds, including antibiotics. Over-expression of efflux pumps resulting in a more efficient antibiotic extrusion. Efflux systems may be responsible for resistance to several chemically distinct antibiotics such as fluoroquinolones, β-lactams, chloramphenicol, and trimethoprim [**21, 26**]
Modification of bacterial molecules that are antimicrobial targets due to mutational events	Structural alterations in an enzyme, primarily caused by mutations located within or near its active site, can inhibit the binding of antibiotics to the target enzyme. Example: Antibiotic resistance in agents targeting enzymes involved in nucleic acid synthesis, such as fluoroquinolones, may arise due to mutations in the genes encoding DNA gyrase or topoisomerase IV. These mutations cause changes in the structure of gyrase and topoisomerase which decrease or eliminate the ability of the antibiotic factor to bind to these components [**20, 21, 24, 27**].
Antibiotic inactivation	Bacteria synthesize enzymes that hydrolyze antibiotics, rendering them ineffective. Example: β-lactamases are enzymes that inactivate β-lactam antibiotics by hydrolyzing a specific bond in the β-lactam ring structure. This structural modification prevents the altered β-lactam antibiotics from binding to their target penicillin-binding proteins (PBPs). The genes encoding β-lactamases can be either inherently located on the bacterial chromosome or acquired through plasmids. The production of β-lactamases is the most prevalent resistance mechanism employed by Gram-negative bacteria against β-lactam antibiotics [**21, 26**]. *PBP: penicillin-binding proteins

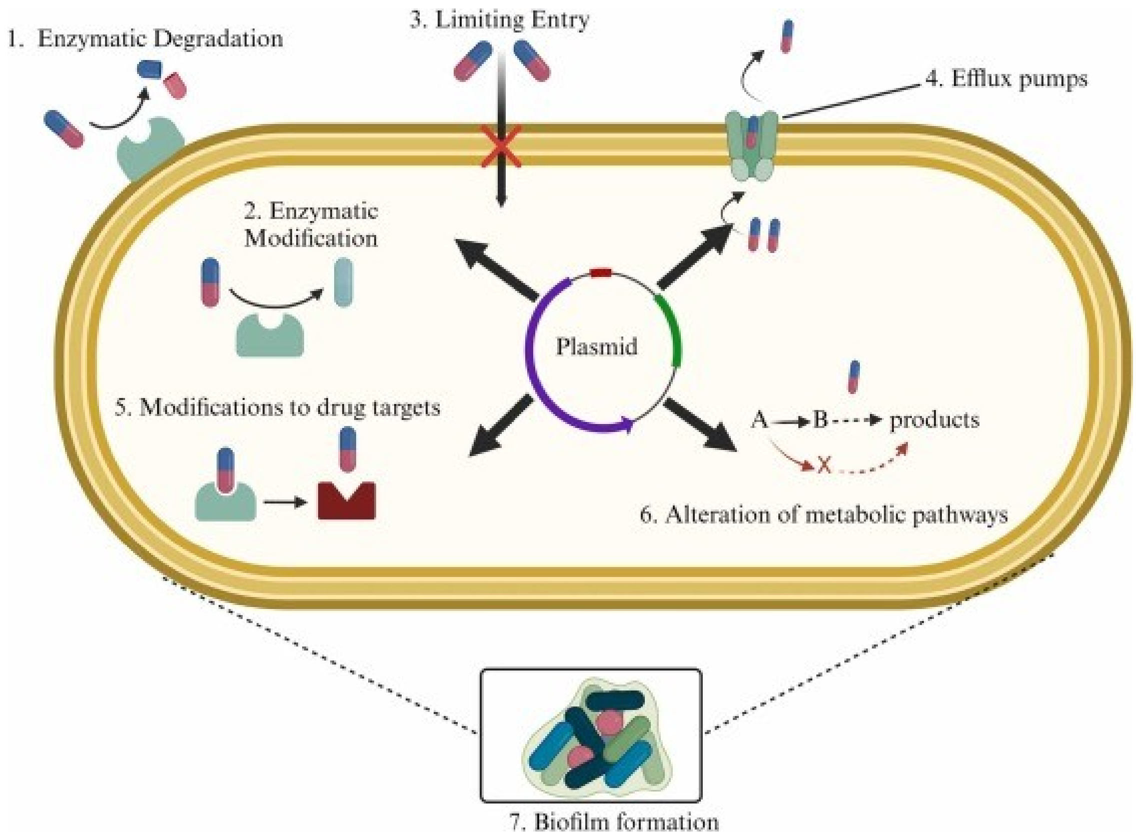


Figure 1.2 The main mechanisms of antibiotic resistance in Gram-negative bacteria [23]

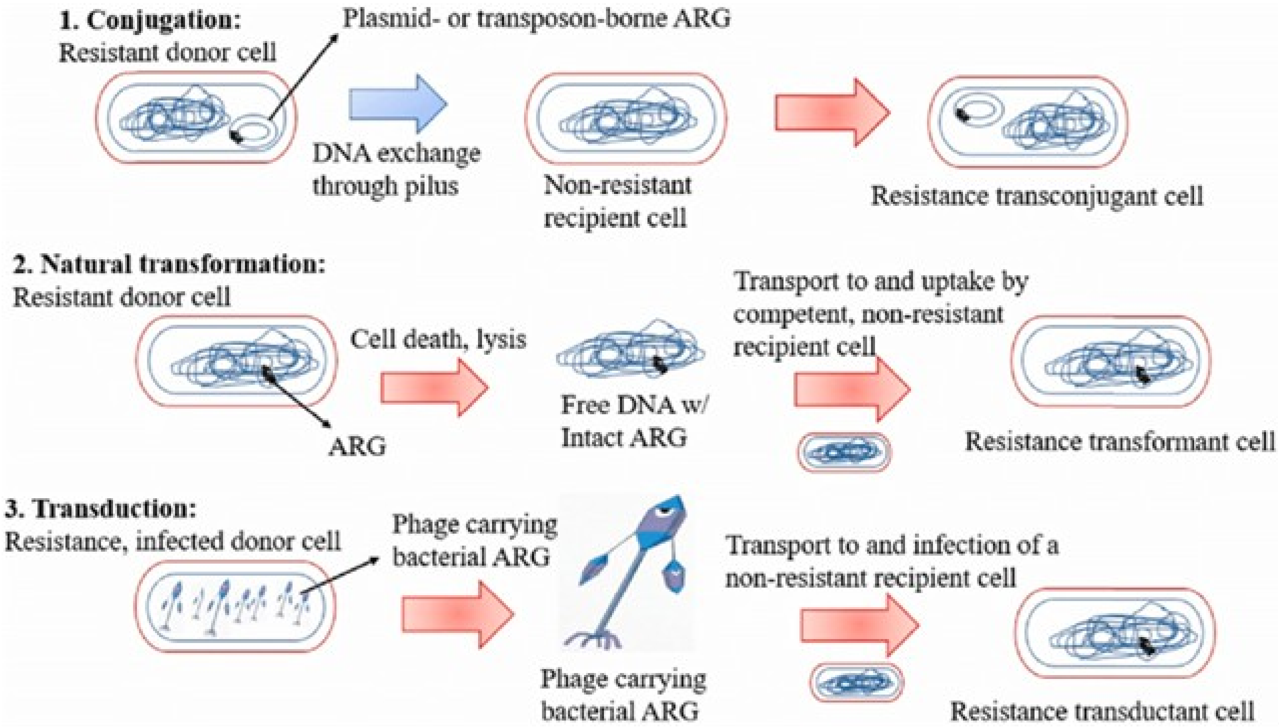


Figure 1.3 Schematic representations of the three mechanisms of HGT [28]

1.4 Mechanisms of resistance to major antimicrobial agents

1.4.1 Resistance to β- lactams: inactivation by β- lactamase enzymes

Extensive use of β-lactams, like other antimicrobial classes, has resulted in the development and spread of resistance. This resistance can arise through various mechanisms, such as changes to the antibiotic's target (through mutation or the expression of alternative penicillin-binding proteins, PBPs), reduced cell permeability due to decreased porin expression needed for β-lactam entry, overproduction of efflux pumps, and the production of enzymes that modify or degrade the antibiotic [**20, 29, 30**]. For β-lactams, resistance often involves enzyme-mediated hydrolysis by β-lactamases, enzymes produced by both Gram-positive and Gram-negative bacteria that hydrolyze the β- lactam ring. In Gram-negative bacteria, β-lactamases remain in the periplasmic space and the genes encoding these enzymes are located either on chromosomes or on plasmids [**20**].

To date, hundreds of different β-lactamase enzymes have been described. While these enzymes share a common function, their amino acid sequences vary, which influences their substrate specificity and inhibitor susceptibility. [**20, 29, 30**]. The identification of an increasing number of β-lactamases, along with the availability of protein and corresponding nucleotide sequence data, has revealed that these enzymes do not form a single homogeneous group but can be categorized into multiple distinct classes [**30, 31**]. B-lactamase enzymes can generally be divided into four types based on their substrate specificity:

Penicillinases: These enzymes specifically target and inactivate penicillin antibiotics.

Narrow spectrum cephalosporinases: These enzymes are more effective against first and second-generation cephalosporins.

Extended-Spectrum β-Lactamases (ESBLs): These enzymes can hydrolyze a broad range of β-lactams, including penicillins, third and fourth- generation cephalosporins, and monobactams.

Carbapenemases: These are the most potent β-lactamases, capable of inactivating even carbapenems.

To achieve a more precise classification of these enzymes, several schemes have been developed. However, the most widely recognized systems for categorizing β-lactamases are those proposed by Ambler and Bush–Jacoby. According to the Ambler classification, β-lactamases are divided into four distinct classes: A, B, C, and D, based on amino acid sequence homology and hydrolytic mechanism. Classes A, C, and D are referred to as serine β-lactamases (SBLs) due to their serine active sites, while class B is known as metallo-β-lactamases (MBLs) or zinc metalloenzymes because of the presence of metal (zinc) ions at their catalytic site [**30**].

In contrast, the Bush–Jacoby system classifies β-lactamases into groups 1 through 4 according to their substrate hydrolysis profiles and their inhibitor profiles, particularly inhibition by β-lactamase inhibitors (such as clavulanic acid and tazobactam) [**32, 33**].

1.4.1a Enzymes that hydrolyze extended-spectrum β-lactams

There are two major families of enzymes that can hydrolyze extended-spectrum β-lactams: extended- spectrum β- lactamases (ESBLs) and AmpC β-lactamases. Enzymes from both families possess the ability to hydrolyze third and fourth-generation cephalosporins, as well as aztreonam. These are potent antibiotics, often used in the treatment of severe, primarily hospital-acquired infections [**31, 32, 33**].

Extended-Spectrum β-Lactamases (ESBL)

ESBL are enzymes that inactivate of broad-spectrum cephalosporins (third- and fourth-generation) and monobactams (aztreonam) but not cephamycins (cefoxitin) or carbapenems (meropenem, imipenem, ertapenem, and doripenem) [**33, 34, 35**] Also, ESBLs are often neutralized by β-lactamase inhibitors (such as clavulanic acid, and tazobactam) [**33, 34, 35**]. ESBLs are produced by diverse range of Gram-negative bacterial species from various families such as *E. coli*, *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp. Among them E. coli is the most common host of ESBLs, followed by *K. pneumoniae* [**36**].

ESBLs are classified under Ambler's classes A and D, where serine functions as the active site of the enzyme. According to the Bush–Jacoby–Medeiros classification system, ESBLs in Ambler's classes A and D are categorized in group 2, specifically in subgroup 2be [**33**]. The most prevalent enzyme families within Ambler class A include TEM-, SHV-, and CTX-M- ESBLs [**34, 37, 38, 39**]. ESBLs have evolved from narrow- spectrum (non- ESBLs, with a more limited range of antibiotic activity) β- lactamases. TEM- type ESBLs are derived from the plasmid- mediated β- lactamase, TEM-1, which was first identified in the early 1960s. TEM-3 was the initial variant to exhibit the ESBL phenotype. Since then, 243 distinct TEM variants have been identified. SHV- type ESBLs, originating from chromosomally encoded enzymes in *K. pneumoniae*, include 228 variants, with SHV-5 and SHV-12 being the most common ESBL enzymes. CTX-M- type β- lactamases were first reported in the late 1980s and were initially named after their ability to hydrolyze cefotaxime. Since the early 2000s, CTX-M- type enzymes have become the most common ESBL group [**37, 38, 39**]. CTX-M enzymes are prevalent in hospital and community settings, as well as in animals, the environment, food products, and livestock. CTX-M enzymes are clustered into five groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 [**38, 39, 40**]. Among the CTX-M- group- 1, the most common enzyme is CTX-M-15, followed by CTX-M-3 and CTX-M-1, while in the CTX-M-9 group, CTX-M-9 and CTX-M-14 are dominant. CTX-M variants efficiently hydrolyze cefotaxime and ceftriaxone (hence the name cefotaximase) and exhibit limited activity against ceftazidime [**37-40**]. However, variants such as CTX-M-27 and CTX-M-15, which have enhanced ceftazidime hydrolytic activity, have been described.

The global spread of ESBL genes is primarily driven by horizontal gene transfer. Most ESBL-encoding genes are plasmid- borne and are associated with various insertion sequences (ISs), including ISEcp1, ISCR1, IS26, and IS10, as well as transposons such as Tn2, and integrons transposons. The plasmids carrying ESBLs are typically conjugative and self-transferred [**27, 37, 38, 39**].

Genes encoding TEM-1, TEM-2, and their ESBL derivatives are usually carried by Tn1-, Tn2-, or Tn3-like transposons, which are embedded in plasmids. The replicon types of conjugative plasmids harboring TEM-type ESBL genes primarily belong to the IncA/C type [**37, 38, 39**].

Genes encoding SHV-type ESBLs can be found either on plasmids or within the chromosome and are often flanked by intact copies of the mobilizing element IS26. Seven plasmid replicon types have been identified that predominantly carry blaSHV-encoding ESBL enzymes, including IncA/C, IncF, IncHI2, IncI1, IncL/M, IncN, and IncX3. Various blaSHV variants have been detected in these plasmid types, with the exception of IncX3, which has only been detected carrying blaSHV-12 [**37, 38, 39**].

Several blaCTX-M types are located adjacent to the mobile element ISEcp1. Elements harboring blaCTX-M are usually carried by conjugative plasmids. For example, blaCTX-M-15 is often embedded in narrow host range plasmids that belong to the IncF replicon type, such as IncFII alone or in association with IncFIA or IncFIB. The dissemination of blaCTX-M group 9 genes appears to be associated with IncHI2-type plasmids, although there have also been reports of IncFII-type plasmids [**27, 37, 38, 39**].

β- Lactamases of class C (AmpC- type lactamases)

Class C β- lactamases, also known as AmpC- type enzymes, can be expressed either from chromosomal or plalasmid- borne genes. Both chromosomal and plasmid- mediated AmpC enzymes confer high-level resistance to cephalosporins, cephamycins (such as cefoxitin), aztreonam, and typically to β-lactamase inhibitors like clavulanic acid. AmpC-type β-lactamases are classified as molecular class C according to the Ambler classification and fall under group 1 in the Bush-Jacoby scheme [**33**].

AmpC- type enzymes are encoded on the chromosomes of many Enterobacteriaceae and other Gram-negative species [**27, 41**]. In several bacterial species, including *Citrobacter freundii*, *Serratia marcescens*, and *P. aeruginosa*, AmpC expression is typically low but can be induced upon exposure to certain β-lactams, mainly to cefoxitin and imipenem. However, derepression of these enzymes - either due to mutation or induced by specific β-lactams - can result in high-level expression, leading to increased resistance to carbapenems, particularly ertapenem. In other organisms, such as *Acinetobacter baumannii* and *E. coli*, one or more components of the induction system are absent [**27, 33, 41**].

Regarding to plasmid-mediated enzymes are also exist in both Enterobacteriaceae and non-fermenting species like *P. aeruginosa*. Plasmid-borne genes encoding certain AmpC family members, such as CMY, ACT, DHA, FOX, and MIR, have been identified. The primary plasmid-encoded AmpC β-lactamases include CMY, DHA, and ACC types, with CMY-type enzymes being the most prevalent worldwide [**27, 40**].

Plasmids carrying AmpC β- lactamase genes commonly belong to incompatibility group IncA/C. These conjugative plasmids often carry additional resistance genes for aminoglycosides, chloramphenicol, quinolones, sulfonamides, tetracycline, and trimethoprim, as well as other β-lactamase genes. Various genetic elements are involved in the mobilization of AmpC genes onto plasmids. For instance, the insertion sequence ISEcp1 is associated with many CMY alleles and is known to facilitate the transposition of adjacent genes, including mobilizing chromosomal β-lactamase genes onto plasmids [**27, 41**].

1.4.1b Carbapenemases

Carbapenemases are β-lactamases that belong to various Ambler classes (A, B, D) and can be encoded by either chromosomal or plasmid-mediated genes. These enzymes are among the most potent β-lactamases, capable of hydrolyzing a wide range of β-lactams, including penicillins, 3rd and 4th generation cephalosporins, aztreonam, and even carbapenems [**33, 42**]. The ability of these enzymes to break down carbapenems, the most potent last-resort antibiotics used in the treatment of very serious infections caused by multidrug-resistant bacteria, is particularly concerning [**36, 38, 40, 42**]. So the clinical significance of carbapenemase production lies in its ability to compromise the efficacy of last-resort antibiotics used for treating severe infections [36, 40]. Epidemiologically, these enzymes pose a major challenge due to their widespread dissemination across various bacterial species and geographical regions [**27, 42, 43**]. Initially, carbapenemase- producing Enterobacteriaceae garnered significant attention following their first report in the early 1990s. More recently, there has been growing concern over the impact of non-fermenting bacteria, such as *A. baumannii* and *P. aeruginosa*, as well as other Gram-negative organisms that produce carbapenemases [**42, 43**].

According to the Bush–Jacoby classification, carbapenemases are categorized into groups 2d, 2f, and 3. Based on the Ambler classification, they are divided into classes A, B, and D. Classes A and D include β-lactamases with serine at their active sites, while class B comprises metalloenzymes that require zinc for their catalytic activity [**33**].

Ambler class A carbapenemases:

Some of these enzymes are encoded on the bacterial chromosome, while others, such as IMI (imipenemase), KPC, and certain variants of GES (Guiana extended spectrum), are plasmid-encoded. Plasmid-encoded enzymes are frequently associated with mobile genetic elements that promote their horizontal transfer between bacteria. Among Ambler class A carbapenemases, KPC (*K. pneumoniae* carbapenemase) is of particular concern due to its presence on self-conjugative plasmids and its frequent association with *K. pneumoniae*, a bacterium known for its capacity to acquire and disseminate resistance genes. The first KPC enzyme, KPC-1, was identified in a *K. pneumoniae* isolate in North Carolina in 1996. Within a few years, KPC-producing strains had spread worldwide, with reports from North and South America, the Middle East, Greece, Italy, and China, where they are now considered endemic **[42, 43].**

Although more than 20 different KPC variants have been identified, KPC-2 and KPC-3 remain the most common. The global dissemination of blaKPC genes in *K. pneumoniae* is associated with the major clone (sequence type ST-258), which serves as a successful transporter. Despite the genetic diversity among KPC variants, blaKPC genes are typically associated to a single transposon, Tn4401. This is a 10-kb Tn3-based transposon, flanked by two 39-bp imperfect inverted repeat sequences, that harbors not only the KPC gene but also a transposase gene, a resolvase gene, and two novel insertion sequences known as ISKpn6 and ISKpn7. The gene blaKPC, except for *K. pneumoniae*, it has also been detected in other Enterobacteriaceae, such as *E. coli*, as well as in *P. aeruginosa* isolates [**27, 42, 43**].

Class B carbapenemases:

These enzymes, known as metallo-β-lactamases (MBLs), require a heavy metal such as zinc for catalysis. MBLs have an extensive substrate spectrum and can hydrolyse virtually all β-lactam antibiotics, including carbapenems, with the exception of monobactams (like aztreonam). Since MBLs are metalloenzymes, they are resistant to commercially available β-lactamase inhibitors (such as clavulanic acid) but are susceptible to inhibition by metal ion chelators like ethylenediaminetetraacetic acid (EDTA) [**33, 27, 42, 43**].

Initially, MBLs were identified as chromosomal enzymes over 50 years ago in environmental and opportunistic pathogenic bacteria, including *Bacillus cereus, Aeromonas spp., Legionella gormanii, Pseudomonas stutzeri, Shewanella spp.,* and *Stenotrophomonas maltophilia*. At present, the most prevalent MBL families, including IMP, VIM, NDM, GIM, and SIM enzymes, are often found within various integrons, where they have been incorporated as gene cassettes. These integrons are embedded in plasmids, allowing them to transfer between bacteria. MBLs are now detected in various Gram-negative bacterial species, where their presence is frequently linked to resistance against multiple antibiotic classes, leading to multidrug resistance and limiting treatment options [**27, 42, 43]**.

The VIM (Verona integron-encoded metallo-β-lactamase) family represents one of the most prevalent groups of MBLs. The VIM β-lactamase gene is carried on a gene cassette within a class 1 integron, conferring resistance to a wide range of β-lactam antibiotics, including ampicillin, carbenicillin, piperacillin, mezlocillin, cefotaxime, cefoxitin, ceftazidime, cefoperazone, cefepime, and carbapenems [**27, 42, 43**]. Although blaVIM genes have been detected in various enterobacterial species, *P. aeruginosa* remains the primary reservoir for these enzymes. To date, over 40 allelic variants of VIM enzymes have been identified, categorized into three major phylogenetic clusters: VIM-1-like, VIM-2-like, and VIM-7-like enzymes. VIM-2-like enzymes are predominantly associated with *P. aeruginosa*, while VIM-1-like enzymes, particularly VIM-4, have been reported in Enterobacteriaceae [**42, 43**].

Another significant MBL gene is NDM (New Delhi Metallo-β-lactamase), which has become a major global concern due to its rapid and widespread dissemination. The first NDM enzyme, NDM-1, was initially identified in a carbapenem- resistant *K. pneumoniae* strain isolated from a urine sample. NDM-1, exhibits only 32.4% similarity to other MBLs such as VIM-1/VIM-2, and can hydrolyze all β-lactams except aztreonam [**33, 27, 42, 43**]. NDM genes are predominantly found in

K. pneumoniae and *E. coli*, but variants have also been identified in *A. baumannii* and *P. aeruginosa*. As of 2020, 24 NDM variants have been identified in over 60 species across 11 bacterial families, with several variants demonstrating enhanced carbapenemase activity. Most blaNDM- carrying plasmids are associated with a few replicon types, including IncX3, IncFII, and IncC [**42, 43**].

Ambler Class D Carbapenemases (Oxacillinases):

Among the earliest detected β- lactamases, class D β-lactamases were relatively uncommon in Enterobacteriae and were always plasmid- mediated [**42, 43, 44**]. These enzymes, often referred to as oxacillinases, are distinguished by their ability to hydrolyze isoxazolylpenicillins such as oxacillin, methicillin, and cloxacillin much more efficiently than classical penicillins like benzylpenicillin, and they show relatively lower activity against first-generation cephalosporins [**33, 42-46**]. The term "OXA" reflects their preference for oxacillin as a substrate [**42**]. The active sites of these enzymes feature a highly conserved serine-based structure, although the rest of the enzyme exhibits variability in amino acid sequences. Notably, OXA enzymes are not inhibited by β-lactamase inhibitors such as clavulanate, sulbactam, or tazobactam, or by metal chelators like EDTA [**33, 42, 44, 45**].

Currently, OXA enzymes with carbapenemase activity include groups such as OXA-23-like, OXA-24/40-like, OXA-48-like, OXA-58-like, OXA-143-like, and OXA-235-like [**42, 43, 44**]. The first carbapenem-resistant OXA-type enzyme identified was OXA-23, found on a large plasmid in a multidrug-resistant *A. baumannii* strain [**42, 43**]. OXA-48-like enzymes are notably prevalent in Enterobacteriaceae and represent a significant concern in carbapenem resistance, with a rising global prevalence over the past decade [**42, 45, 46**]. OXA-48 exhibits low-level hydrolytic activity against carbapenems, with greater activity against imipenem compared to meropenem, and only modest hydrolysis of expanded-spectrum cephalosporins such as ceftazidime and cefepime [**42, 45, 46**]. Despite this, combined with poor permeability, it can result in high-level carbapenem resistance. OXA-48 primarily hydrolyzes penicillins and narrow-spectrum cephalosporins [**33, 42, 45]**. However, Enterobacteriaceae harboring blaOXA-48-like genes may also carry genes encoding ESBLs (blaCTX-M, blaSHV, blaTEM) or AmpC enzymes, which contributes to resistance to aztreonam, extended-spectrum cephalosporins, and carbapenems **[42, 45, 46**]. The detection of OXA-48-like producers can be challenging, as the level of acquired carbapenem resistance may be low, leading to underreporting of these strains.

OXA-48 is now widespread not only in *K. pneumoniae* but also in other Enterobacteriaceae. Reports of OXA-48 producers have been sporadic across various European countries, including France, Germany, the Netherlands, Italy, Belgium, the UK, Ireland, Slovenia, Switzerland, and Spain [**42, 45**].

The primary vector for the blaOXA-48 gene is the 62.3 kb plasmid pOXA-48a, which belongs to the IncL/M type. This plasmid has integrated the gene via the Tn1999 composite transposon, which includes IS1999 insertion sequences that promote blaOXA-48 gene expression [**42, 43, 45, 46**]. The current spread of OXA-48 producers is thus linked to this highly conjugative plasmid. Nevertheless, chromosomal integration of OXA-48 has been documented in *E. coli* strains from the UK and Egypt [**42**]. Variants of OXA-48 include OXA-48, OXA-181, OXA-232, OXA-204, OXA-162, OXA-163, and OXA-244, with these being the most common among the group [**42, 43, 45, 46]**.

1.4.2 Resistance to quinolones

Quinolones and fluoroquinolones (next- generation quinolones) are critical antimicrobial agents used to treat a wide range of infections in humans and animals [**20, 27**]. These agents are effective in killing almost all types of bacteria. Resistance to quinolones and fluoroquinolones generally arises from mutations in the genes encoding DNA gyrase and topoisomerase IV, which are the primary drug targets [**20, 27**]. Additionally, other resistance mechanisms, such as decreased outer membrane permeability, protection of target structures, or increased activity of efflux pumps, may also contribute to reduced drug efficacy [**20, 27**].

1.4.2a Chromosomal resistance to quinolones: drug-target modification

The main target of (fluoro)quinolones is DNA gyrase, which is composed of two GyrA and two GyrB subunits. Additionally, topoisomerase IV serves as a secondary target in Gram-negative bacteria, consisting of two ParC and two ParE subunits [**20, 27**]. Mutations related to quinolone resistance are predominantly found in the quinolone resistance- determining region (QRDR) of the GyrA subunit, specifically between Ala67 and Gln107, with the most frequent mutations occurring at codons 83 and 87 [**27**]. While single mutations in gyrA can lead to resistance to quinolones, fluoroquinolone resistance typically requires additional mutations in gyrA and/ or parC. Most mutations in parC are found at codons 80 and 84 [**27**].

1.4.2b Plasmid-mediated resistance to quinolone: target protection, enzymatic modification, and efflux pumps

Since the discovery of the first plasmid-mediated quinolone resistance (PMQR) gene, qnrA1, in 1997, there has been growing concern about the global spread of PMQR genes **[20, 27**]. Various plasmid-encoded mechanisms of resistance have been identified, including: i) Qnr-like proteins (QnrA, QnrB, QnrC, QnrD, and QnrS), which shield DNA from the effects of quinolones; ii) the AAC(6′)-Ib-cr acetyltransferase, which modifies specific fluoroquinolones (such as ciprofloxacin); and iii) active efflux pumps such as QepA and OqxAB **[20, 27**]. These resistance factors typically do not induce high- level resistance to quinolones or fluoroquinolones, but they confer reduced susceptibility to these drugs. Additionally, they may facilitate the emergence of strains with higher resistance levels through the interaction with other chromosomally encoded resistance mechanisms [**20, 27**].

Plasmid-mediated quinolone resistance (PMQR) genes have been widely identified in both human and animal isolates. In Europe, the most commonly detected genes in *E. coli* isolates are qnrS1 and qnrB19, as well as qnrB1, qnrB4, and qnrB10 [**20, 27**]. The PMQR genes qnrS1 and qnrB19 are often associated with plasmids belonging to the IncN and IncX replicon types, among others. The aac(6′)Ib-cr gene was found on plasmids belonging to the IncF family, frequently alongside a blaCTX-M ESBL gene [**27**].

1.4.3 Resistance to sulfonamides and trimethoprim

Sulfonamides and trimethoprim are synthetic antimicrobial drugs that target different stages in the folic acid synthesis pathway [**20, 27**]. Each of these agents works by inhibiting bacterial growth (bacteriostatic effect), but when combined, they produce a synergistic bactericidal effect on susceptible bacteria [**20**]. These drugs have been widely used in both animals and humans for decades. Acquired resistance mechanisms have been frequently identified, mainly due to: i) mutations in the genes encoding the target enzymes, such as dihydropteroate synthase for sulfonamides or dihydrofolate reductase for trimethoprim or ii) the acquisition of sul genes which encode dihydropteorate synthetases that are insensitive to sulfonamides or dfr genes which encode dihydrofolate reductases that are insensitive to trimethoprim [**20, 27]**.

Regarding sul genes, resistance to sulfonamides can be conferred by any of the three sul genes: sul1, sul2, or sul3 [**27**]. The sul1 gene is especially widespread as it is part of the 3′-conserved segment of class 1 integrons (**Figure 1.4**), which are often found on plasmids that also carry additional resistance genes [**27**].

As for the dfr genes that provide resistance to trimethoprim, they have been identified in Enterobacteriaceae and other Gram-negative bacteria [**27**]. Based on their size and structure, these genes are categorized into two main groups, dfrA and dfrB. Most dfrA and dfrB genes are found on gene cassettes that are inserted into class 1 (**Figure 1.4**) or class 2 integrons [**27**].

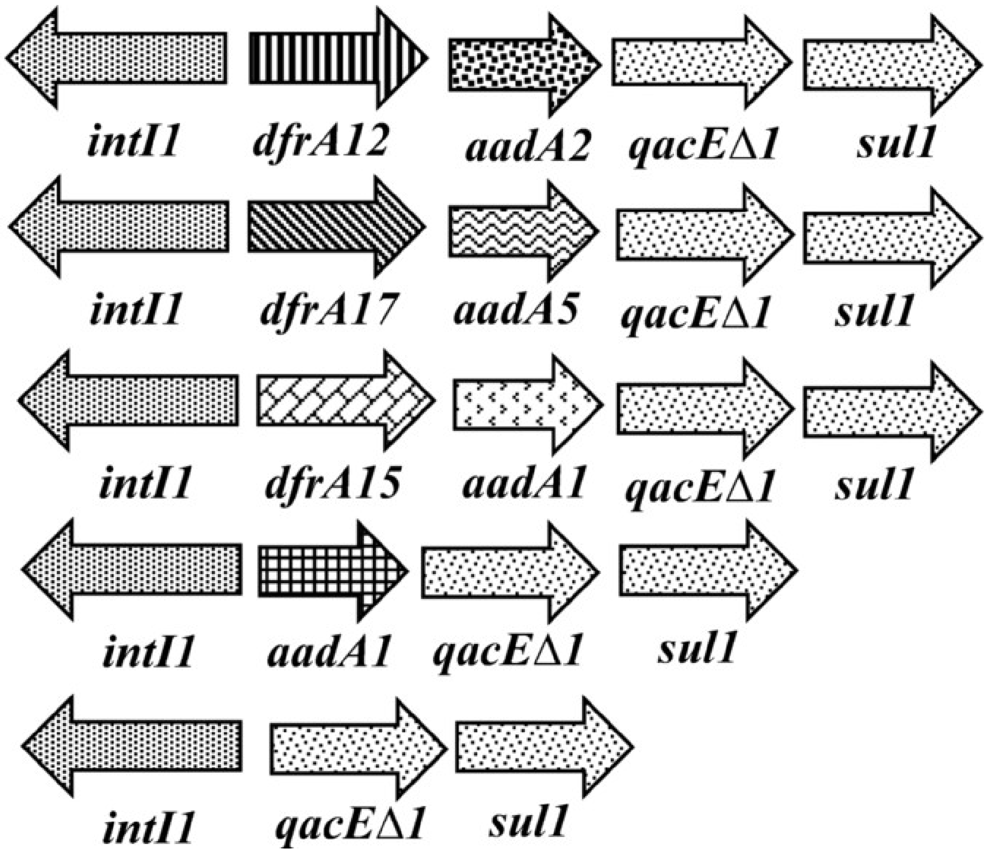


Figure 1.4 Schematic diagrams showing a gene cassette with multiple resistance genes

1.4.4 Colistin resistance mechanisms

Colistin resistance has become a significant concern in recent years, particularly because this antibiotic is often considered a last- resort treatment for infections caused by multidrug- resistant gram-negative bacteria [**20, 27**]. The mechanisms involved in colistin resistance are both chromosomal and plasmid- mediated [**27, 47**]. However, the plasmid-mediated mechanisms are of greater concern because they can be easily transferred, spreading resistance among bacterial populations [**20, 27, 47**].

1.4.4a Plasmid-mediated colistin resistance

The most common mechanism of colistin resistance in Enterobacteriaceae involves the presence of mobilized colistin resistance (mcr) genes [**27, 49**]. Since the discovery of mcr-1 in 2015, several variants have been identified, including mcr-2, mcr-3, mcr-4, mcr-5, mcr-6, and more [**48, 49, 50, 51, 52**]. These genes are typically located on conjugative plasmids and encode enzymes that modify the bacterial cell membrane by adding phosphoethanolamine to the lipid-A component of lipopolysaccharides (LPS) [**27, 47**]. This modification reduces the binding affinity of colistin to the bacterial membrane, rendering the antibiotic less effective [**27**]. The first mcr genes were detected in Salmonella spp and *E. coli* strains isolated from animal samples, primarily poultry and pigs [**48-52**]. Now, there are also reports of mcr in Enterobacteriaceae from human samples, as well as from environmental samples such as river water **[27**, **48-52**]. The spread of these plasmid-mediated genes in clinical settings poses significant challenges for infection control

1.4.4b Colistin resistance due to chromosomal gene mutations

Chromosomal Mutations: Resistance can also arise from mutations in chromosomal genes, such as those encoding the two-component regulatory systems (e.g., pmrA/pmrB), which alter the lipid A component of the bacterial outer membrane [**20, 27, 49**].

1.5 Current state of AMR and impacts

The emergence of AMR to nearly all clinically relevant antibiotics is a pressing health risk issue that could reverse a century of medical progress [**53, 54, 55**]. AMR exists everywhere and threatens not only human health but also animal health, the environment, food and nutrition security and safety, as well as economic development [**56, 57, 58, 59]**. For the above reasons, the World Health Organization (WHO) lists AMR among the top 10 threats for global health. [**53, 54, 55, 56, 57, 58, 59**]

AMR hampers the effectiveness of antibiotic treatments, making previously manageable infections increasingly difficult to treat [**53, 54, 55, 57**]. This growing challenge is leading to higher rates of illness and death worldwide [**2, 23, 56, 57**]. As first- line antimicrobials lose their effectiveness; there is an increasing reliance on second- and third-line therapies. In severe cases, last-resort antibiotics like carbapenems are required to combat multidrug- resistant infections. [**23, 56, 57**]

AMR has far-reaching consequences, not only for individual patients but also for public health. For patients, the absence of effective antibiotics often means longer recovery times, or in some cases, no treatment options at all, potentially resulting in death [**23, 56, 57, 58**]. At the community level, this situation increases the risk of infection outbreaks, epidemics, and even pandemics. Furthermore, routine medical procedures such as surgeries, organ transplants, chemotherapy, and neonatal care are becoming increasingly hazardous due to the diminished ability to control infections [**23, 56, 59**].

It was estimated that bacterial AMR contributed to approximately 4.95 million (3.62–6.57 million) deaths in 2019, with 1.27 million (95% UI 0.911–1.71 million) of those deaths directly attributed to bacterial AMR [**60**]. Regionally, western sub-Saharan Africa had the highest death rate due to resistance, with 27.3 deaths per 100,000 people (20.9–35.3), while Australasia had the lowest at 6.5 deaths per 100,000 people (4.3–9.4). Lower respiratory infections were the leading cause, responsible for over 1.5 million deaths linked to resistance in 2019, making them the most burdensome infection type **(Figure 1.5) [60]**.

Resistant strains of the six major pathogens - *E. coli*, methicillin- resistant *Staphylococcus aureus* (MRSA), *K. pneumoniae*, *Streptococcus pneumoniae*, *A. baumannii*, and *P. aeruginosa* - were responsible for 929,000 (660,000–1,270,000) deaths attributable to AMR (**Figure 1.6**) and 3.57 million (2.62–4.78 million) deaths associated with AMR in 2019 [**23, 36, 56, 60].** Among these, MRSA alone caused over 100,000 deaths attributable to AMR, while six additional pathogen-drug combinations, including multidrug-resistant tuberculosis (excluding extensively drug-resistant forms), third-generation cephalosporin-resistant *E. coli* **(Figure 1.7)**, carbapenem-resistant *A. baumannii*, fluoroquinolone-resistant *E. coli*, carbapenem-resistant *K. pneumoniae*, and third-generation cephalosporin-resistant *K. pneumoniae*, each caused between 50,000 and 100,000 deaths [**23, 55, 56, 58, 60**]

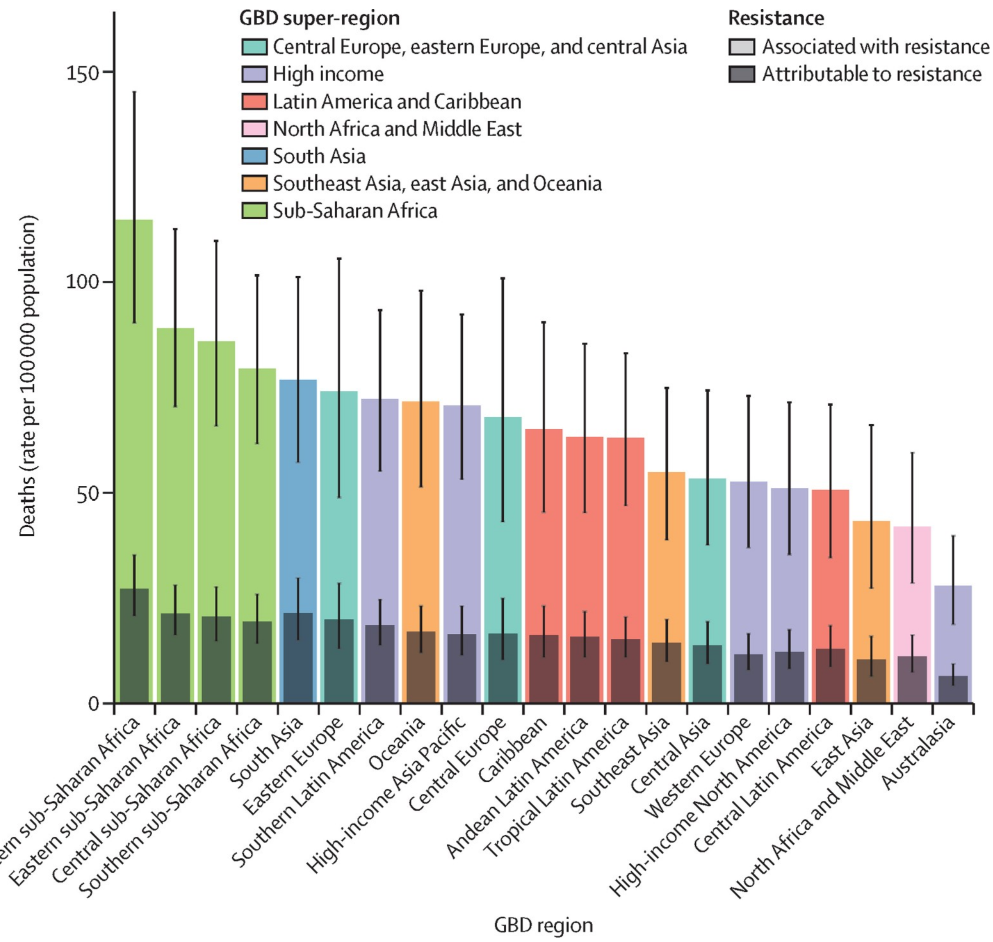


Figure 1.5 All-age rates of deaths attributable to and associated with bacterial antimicrobial resistance by GBD region, 2019 [60]

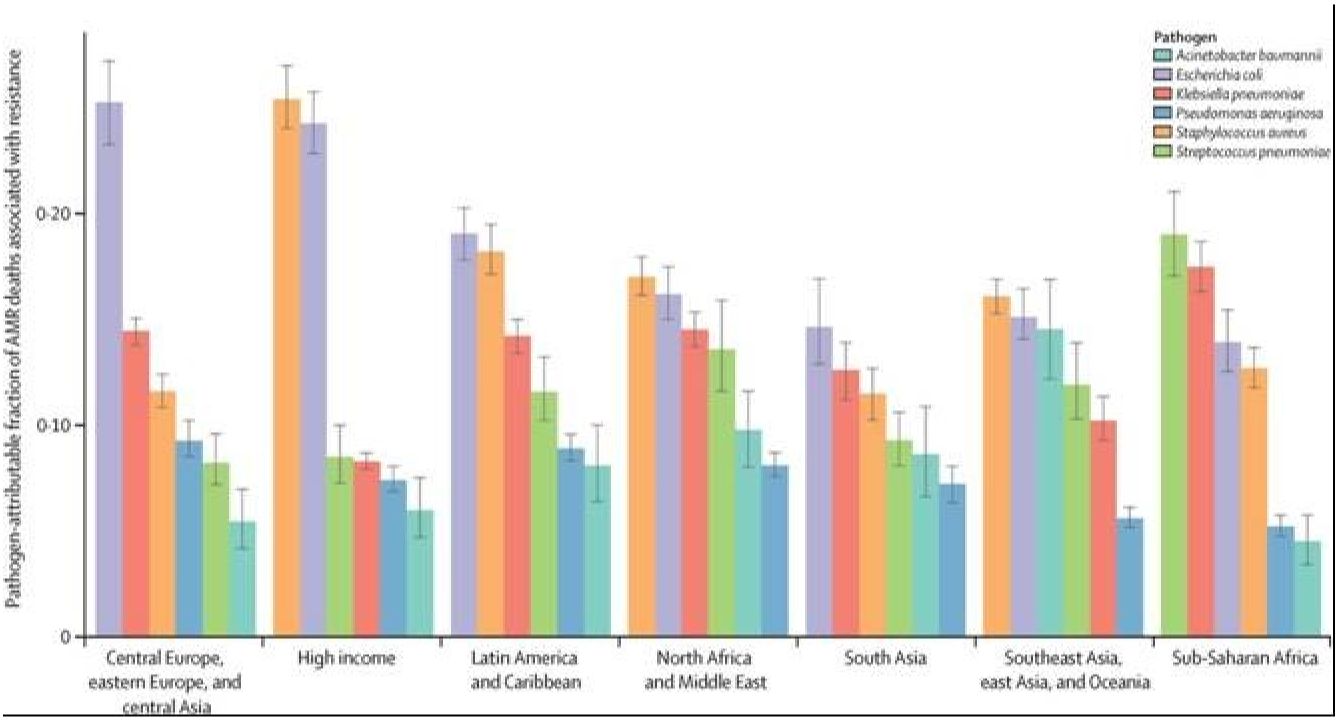


Figure 1.6 Pathogen-attributable fractions of deaths attributable to bacterial AMR for the six leading pathogens by GBD super-region, 2019 [60]

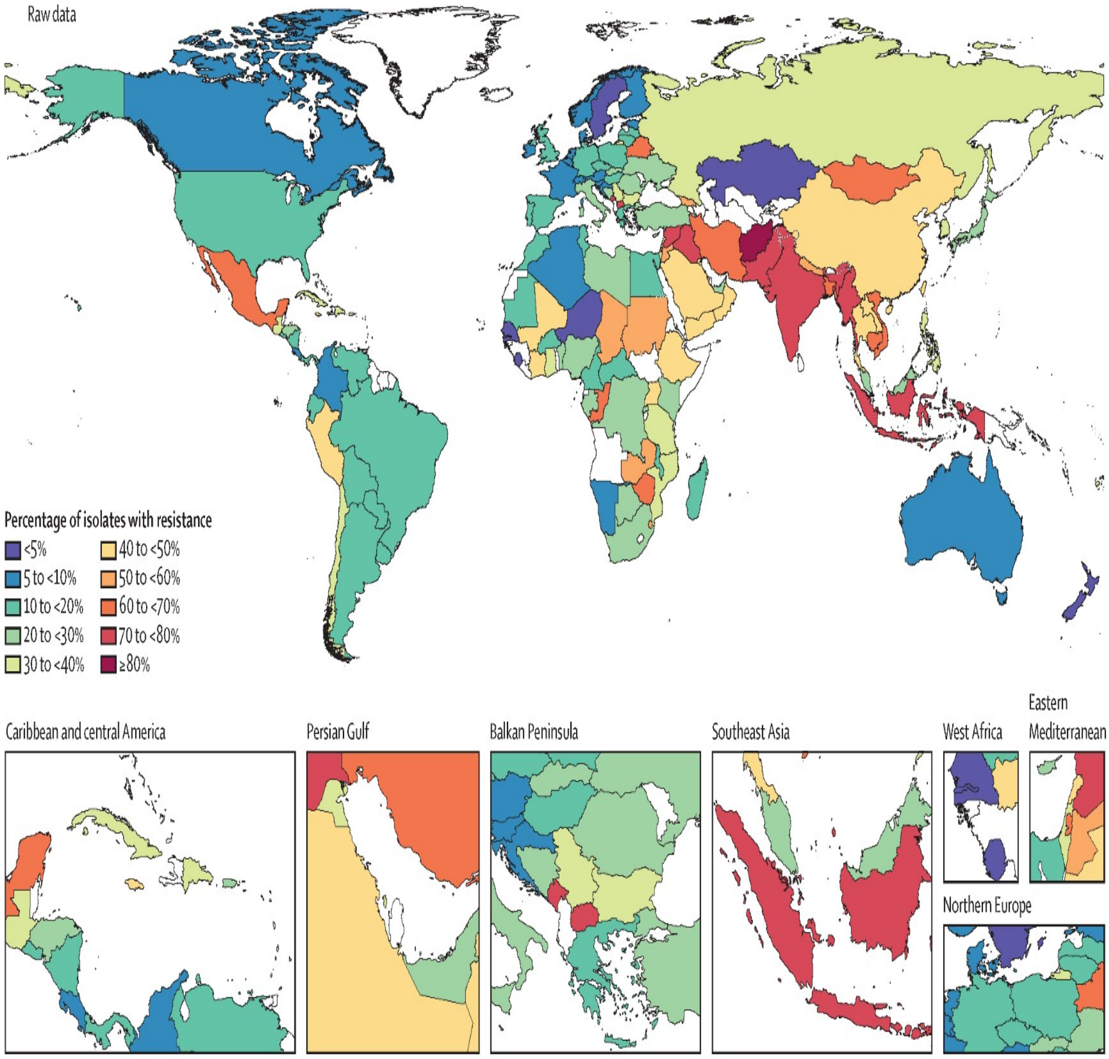
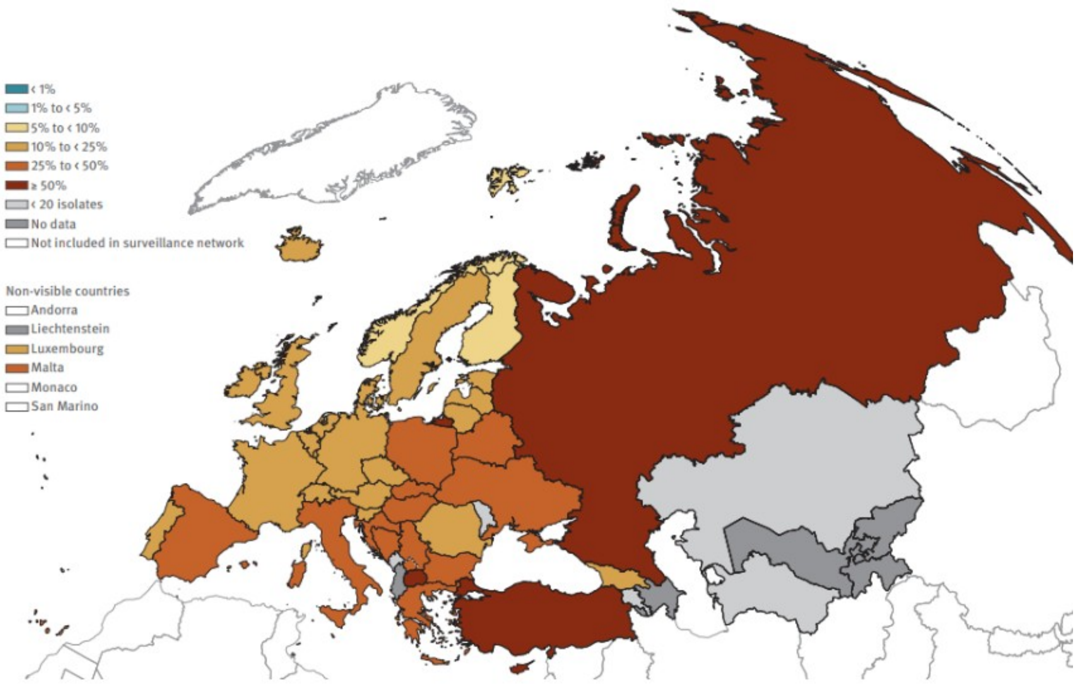


Figure 1.7 The raw data for third-generation cephalosporin-resistant E. coli by country and territory in 2019 [60]

1.5.1 Current state of AMR and impacts in Europe

In Europe, the situation is particularly concerning, with reports indicating alarmingly high AMR rates, especially in southern and eastern regions. According to the 2021 data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) [**61**], several countries reported resistance rates reaching or exceeding 25%, and in some cases, over 50% for last-resort antibiotics like carbapenems in pathogens such as *K. pneumoniae*, *P. aeruginosa*, and Acinetobacter spp [**61**]. Specifically, for *E. coli* - the most common cause of urinary tract and bloodstream infections- the highest European union (EU) population- weighted mean resistance was reported for aminopenicillins (53.1%), followed by fluoroquinolones (21.9%), and third-generation cephalosporins (13.8%), as shown in the maps in **Figures 1.8a and b [61]**. Although carbapenem-resistant *E. coli* isolates remain rare, a small but significant increase was noted between 2017 and 2021[**61**].

a)



b)

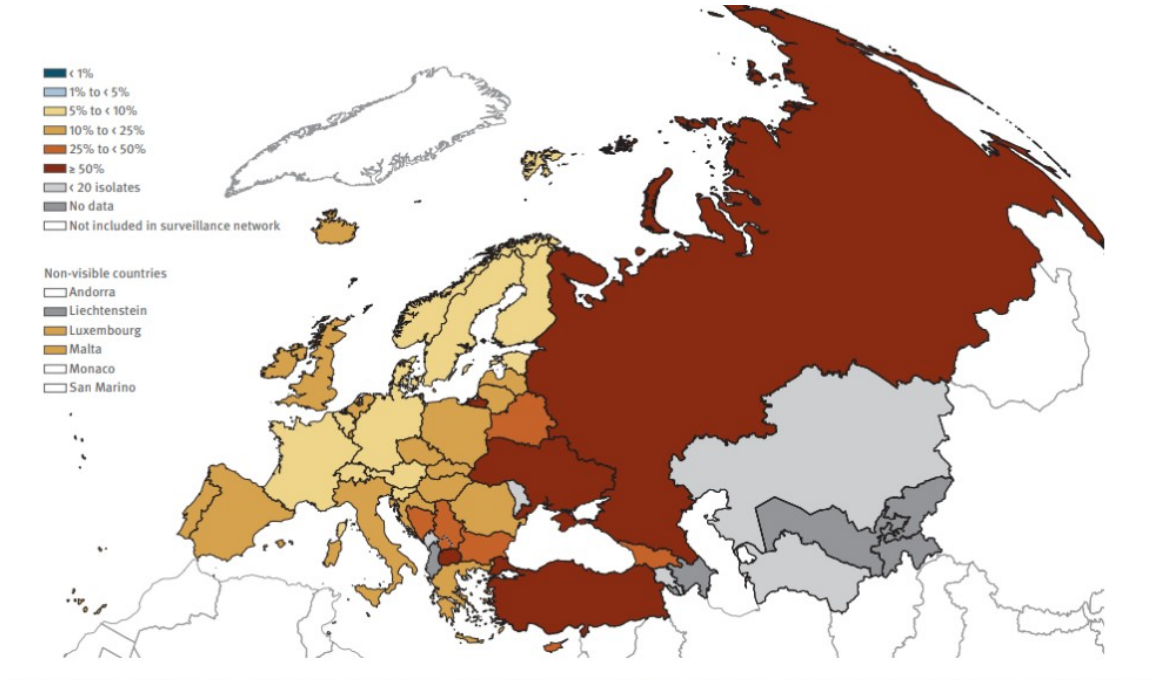


Figure 1.8 Maps showing the percentages of invasive E. coli* strains *resistant to (a) fluoroquinolones and (b) third-generation cephalosporins, by country, in Europe in 2021 [61].

The European Centre for Disease Prevention and Control (ECDC) conducted a study on the health burden of AMR in the EU/EEA from 2016 to 2020 [**62**]. The study found that the greatest disease burden came from infections with third-generation cephalosporin- resistant *E. coli*, followed by MRSA and third-generation cephalosporin-resistant *K. pneumoniae* (**Figure 1.9**) [**62**]. In 2020, carbapenem- resistant *K. pneumoniae* was estimated to have caused 4,076 deaths. These findings highlight the urgent need for ongoing monitoring and enhanced efforts to effectively address this public health threat **[61, 62].**

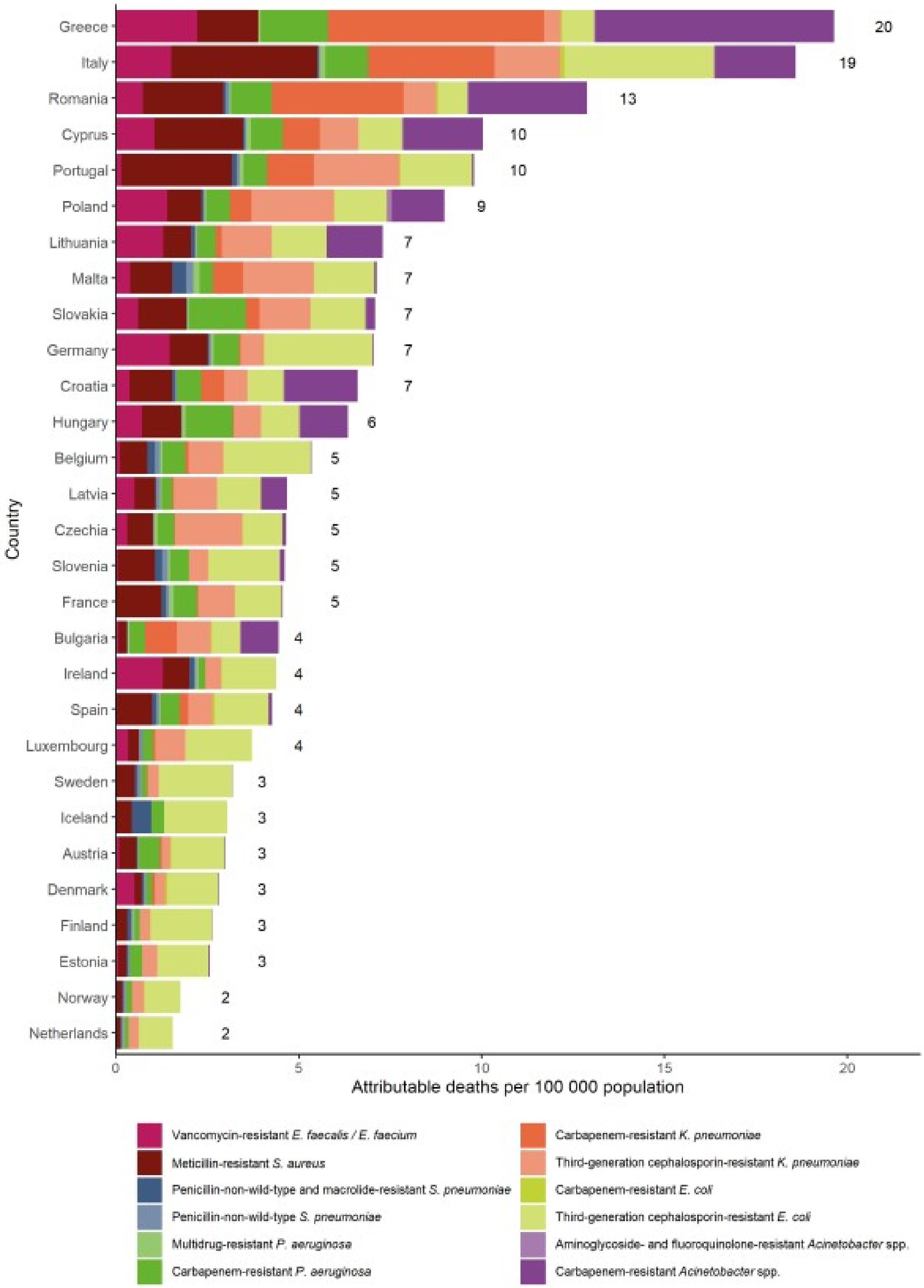


Figure 1.9 Estimations of the burden of infections with antibiotic-resistant bacteria presented as attributable deaths per 100 000 population by country, EU/EEA, 2020 [62]

AMR to critical antibiotics in zoonotic bacteria is an escalating threat, particularly with pathogens like *E. coli*, Salmonella spp., and Campylobacter spp. This resistance facilitates transmission to humans, whether through the food chain or direct contact, and significantly endangers public health. The European Food Safety Authority (EFSA) report for 2021-2022 provides valuable insights into the resistance patterns of these zoonotic bacteria [**63**]. Notably, resistance to fluoroquinolones, particularly ciprofloxacin, is alarmingly high among Campylobacter isolates from poultry [**63**]. This is concerning given the role of these bacteria in foodborne illnesses. In addition, the report highlights the prevalence of ESBL-/AmpC-producing isolates in broilers, with resistance rates ranging from 24.6% in Latvia to 97.7% in Germany. In cattle, these rates ranged from 16.7% in Denmark to 98.5% in Germany [**63, 64, 65**] (**Figures 1.10a, b**). These findings underscore the widespread nature of AMR in food-producing animals across Europe.

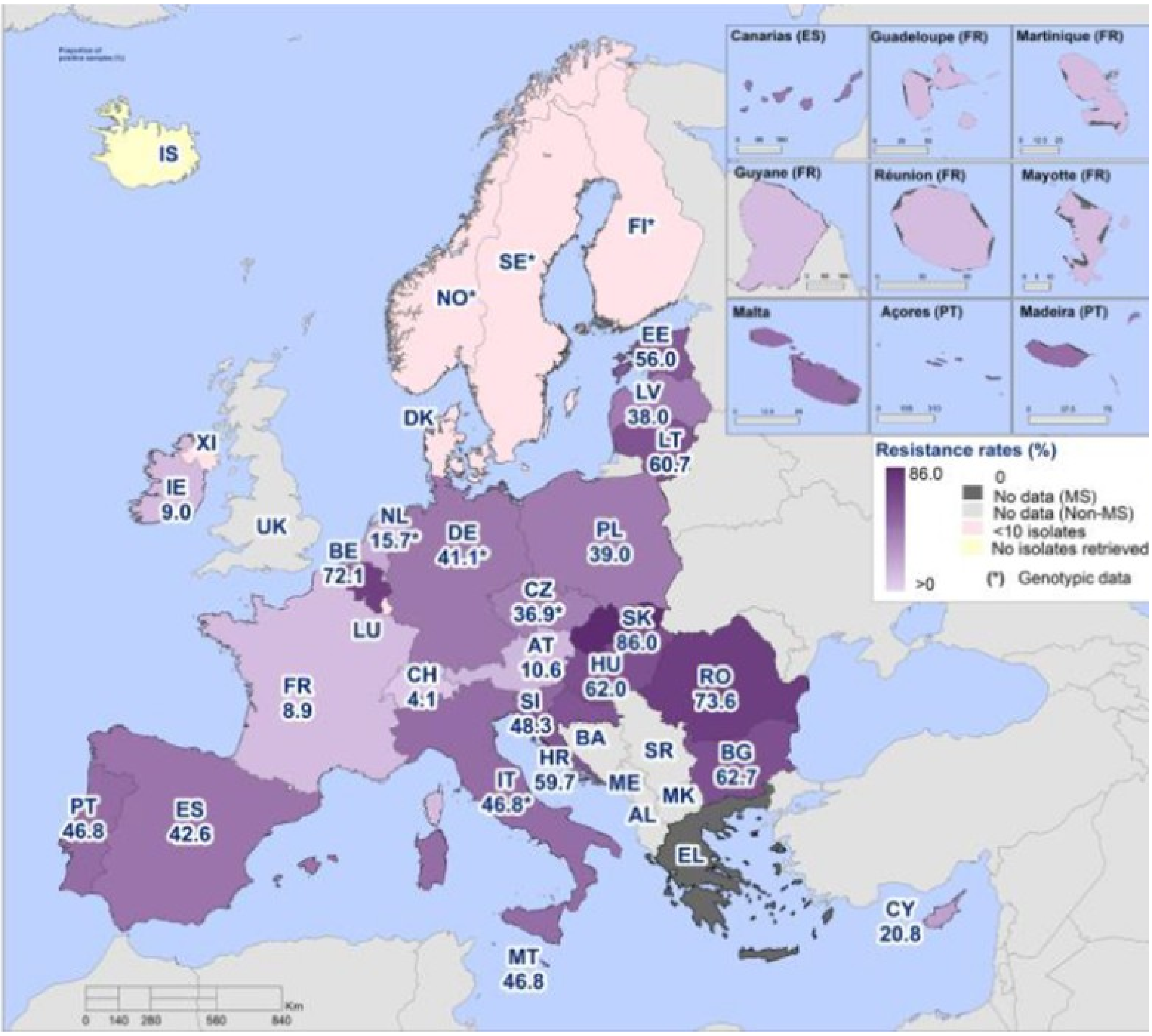
Resistance to last-resort antibiotics, such as colistin and tigecycline, is particularly worrisome [**63**]. While colistin resistance in *E. coli* from food-producing animals remains relatively low, its presence is still a concern given its critical role in treating multidrug-resistant infections [**63**]. Furthermore, some countries have reported very high levels of resistance to tigecycline—another last-resort antibiotic used to treat serious infections caused by MDR bacteria—in Salmonella isolates from broilers [**63**].

The increasing resistance to fluoroquinolones in *Salmonella Enteritidis* and *Campylobacter jejuni* isolates, both commonly associated with poultry, is a significant public health concern [**63**]. In severe cases of infection, fluoroquinolones are among the key antimicrobials used for treatment, making this trend particularly alarming [**63**].

Beyond food-producing animals, AMR has also been observed in companion animals. Studies [**64, 65**] have identified enterobacterial strains resistant to carbapenems in pets (**Figure 1.11**).

The restricted treatment options due to AMR in livestock not only pose a threat to animal health but also increase the risk of outbreaks among cattle, poultry, and sheep [**58, 59**]. This often necessitates culling, leading to significant economic losses and threatening food security. It is estimated that AMR could impose a $3–4 billion financial burden on the livestock sector alone in the coming decades [**58, 59**].

The economic impact of AMR is difficult to quantify due to the various factors involved. Increased resistance leads to higher costs associated with more expensive antibiotics, specialized equipment, prolonged hospitalization, and isolation procedures for patients [**58, 59**]. In Europe, the overall economic burden of antibiotic resistance is estimated to be at least 1.5 billion euros, with over 900 million euros attributed to hospital costs [**23, 58, 59, 62]** a)



Canarias (ES)
Guadeloupe (FR)
Martinique (FR)
Guyane (FR)
Réunion (FR)
Mayotte (FR)
Malta
Açores (PT)
Madeira (PT)
Resistance rates (%)
86.0
>0
0
No data (MS)
No data (Non-MS)
<10 isolates
No isolates retrieved
(*) Genotypic data
IS
NO*
SE*
FI*
EE
56.0
LV
38.0
LT
60.7
DK
XI
IE
9.0
UK
NL
15.7*
BE
72.1
LU
DE
41.1*
PL
39.0
CZ
36.9*
SK
86.0
AT
10.6
HU
62.0
RO
73.6
CH
4.1
FR
8.9
SI
48.3
BA
SR
BG
62.7
HR
59.7
ME
MK
AL
IT
46.8*
PT
46.8
ES
42.6
EL
CY
20.8
MT
46.8
Km
0 140 280 560 840

b)

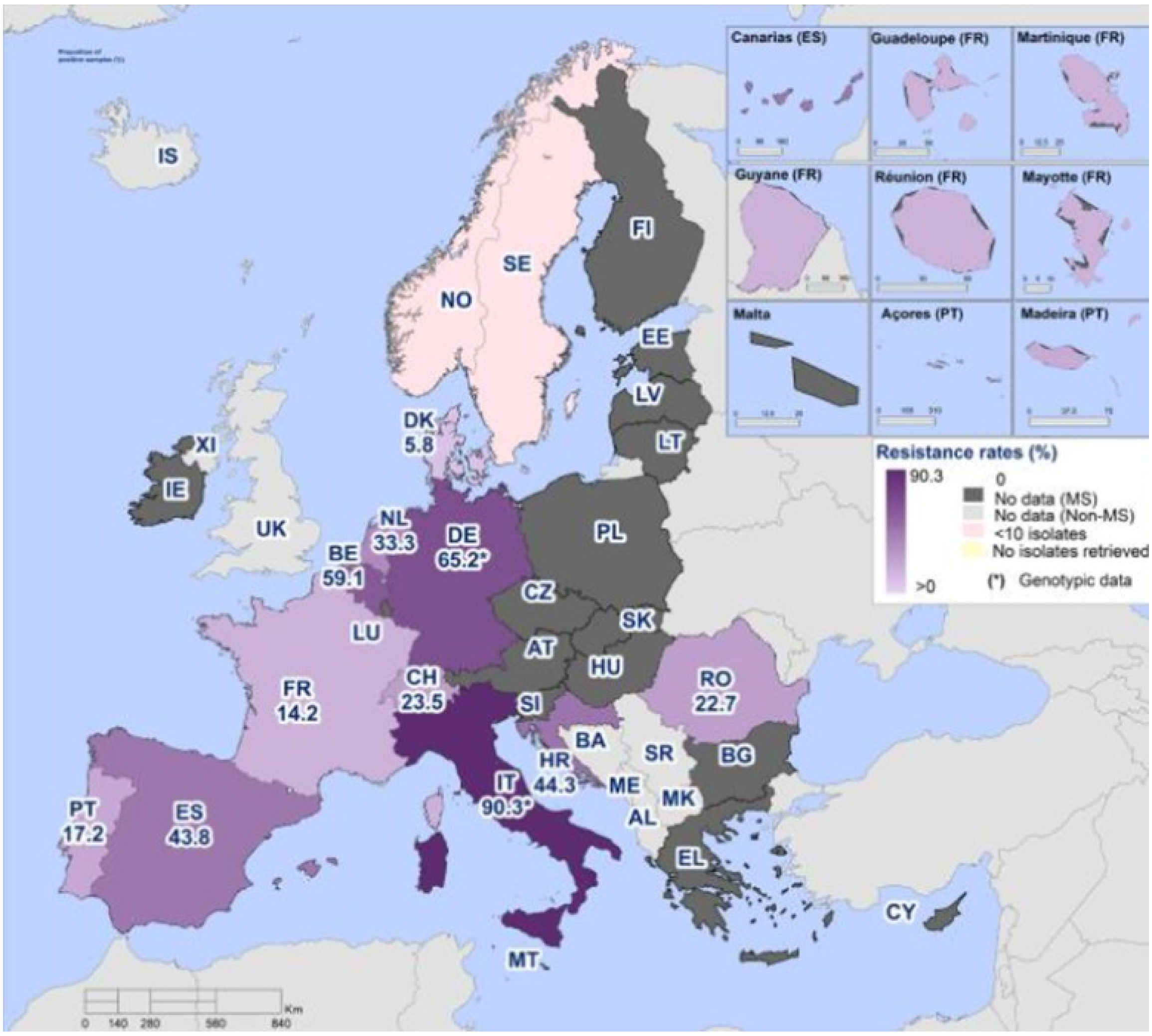


Figure 1.10 Spatial distribution of the prevalence of presumptive ESBL- and/or AmpC- producing Escherichia coli from (a) cattle under 1 year of age, (b) broilers, EU MSs and non-MSs, 2021/2022 [63].

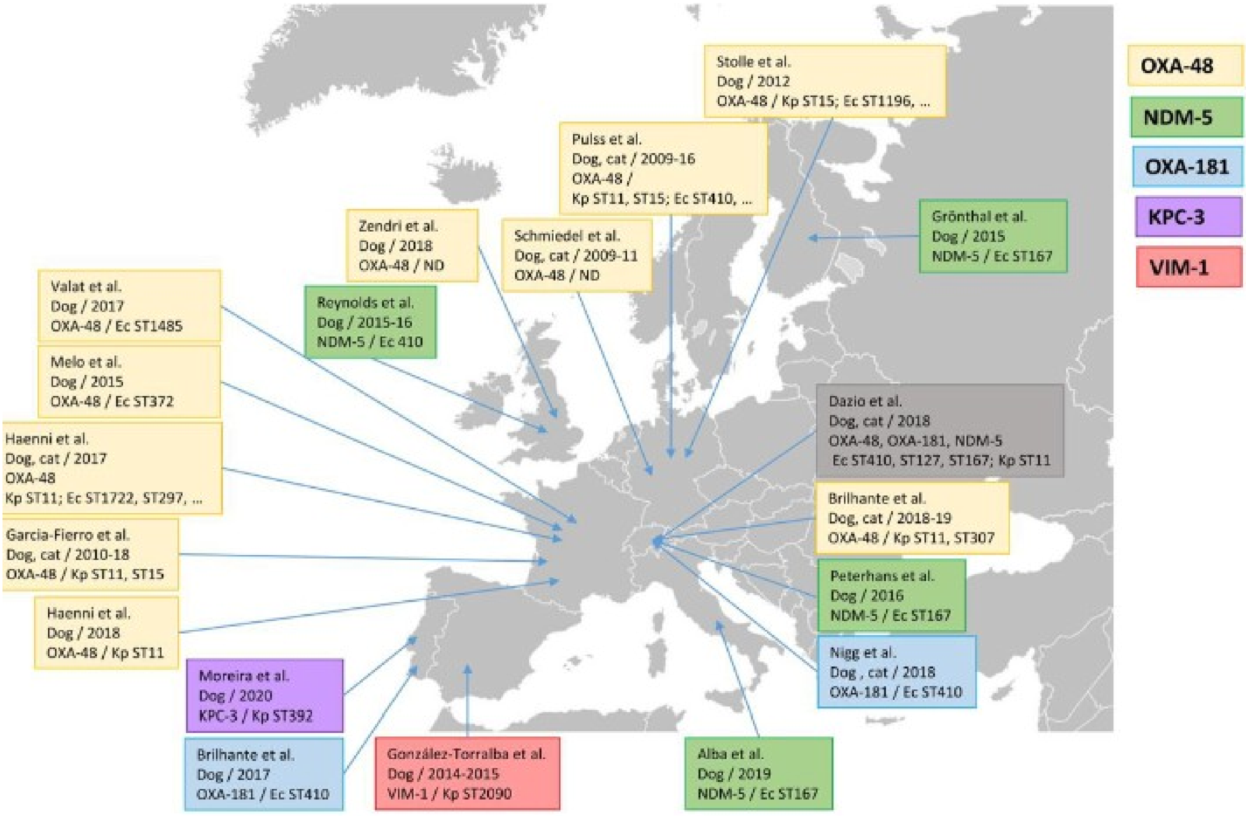


Figure 1.11 Reported carbapenem- resistant Enterobacterales in companion animals [63]

1.5.1a Current state of AMR in Greece

Greece is one of the most affected by the AMR countries in Europe. The high prevalence of resistant bacteria, particularly in hospital settings, poses a significant challenge to public health and healthcare systems. Greece's situation reflects broader trends in the Mediterranean region, where antibiotic overuse and misuse have contributed to the rapid emergence and spread of resistant pathogens [**61, 62**].

Greece consistently reports some of the highest rates of AMR in Europe. Our country faces significant challenges with resistance to several critical classes of antibiotics, particularly in hospital-acquired infections (HAIs) [**61, 62**]. In 2021, according to surveillance report published by the European Centre for Disease Prevention and Control (ECDC), Greece is among the countries with the highest rates of invasive Gram-negative bacteria such as *K. pneumoniae* and Acinetobacter spp, with resistance to carbapenems [**61**]. **Figure 1.12** shows a significant increase in nosocomial infections caused by carbapenem- resistant isolates of *K. pneumoniae* and *Acinetobacter* spp in 2020, as well as an increase in infections caused by third- generation cephalosporin- resistant iolates of *E. coli* and *K. pneumoniae* [**61**]. Additionally, Greece recorded the highest death rate attributable to AMR, with 20 deaths per 100 000 people in 2020 [**62, 66**] (see **Figure 1.9**).

Furthermore, based on annual epidemiological report for the year 2022 issued by the European Surveillance of Antimicrobial Consumption Network (ESAC-Net), Greece also ranks first in antibiotic consumption, both in hospital settings and in community [**67**]. Specifically, the community antibiotic consumption rate is 32.1% (**Figure 1.13) [67]**. As for hospital sector, Greece is among the countries that have seen statistically significant increases in the consumption of carbapenems and polymyxins over the past 10 years (**Figure 1.14)** [**66, 67**].

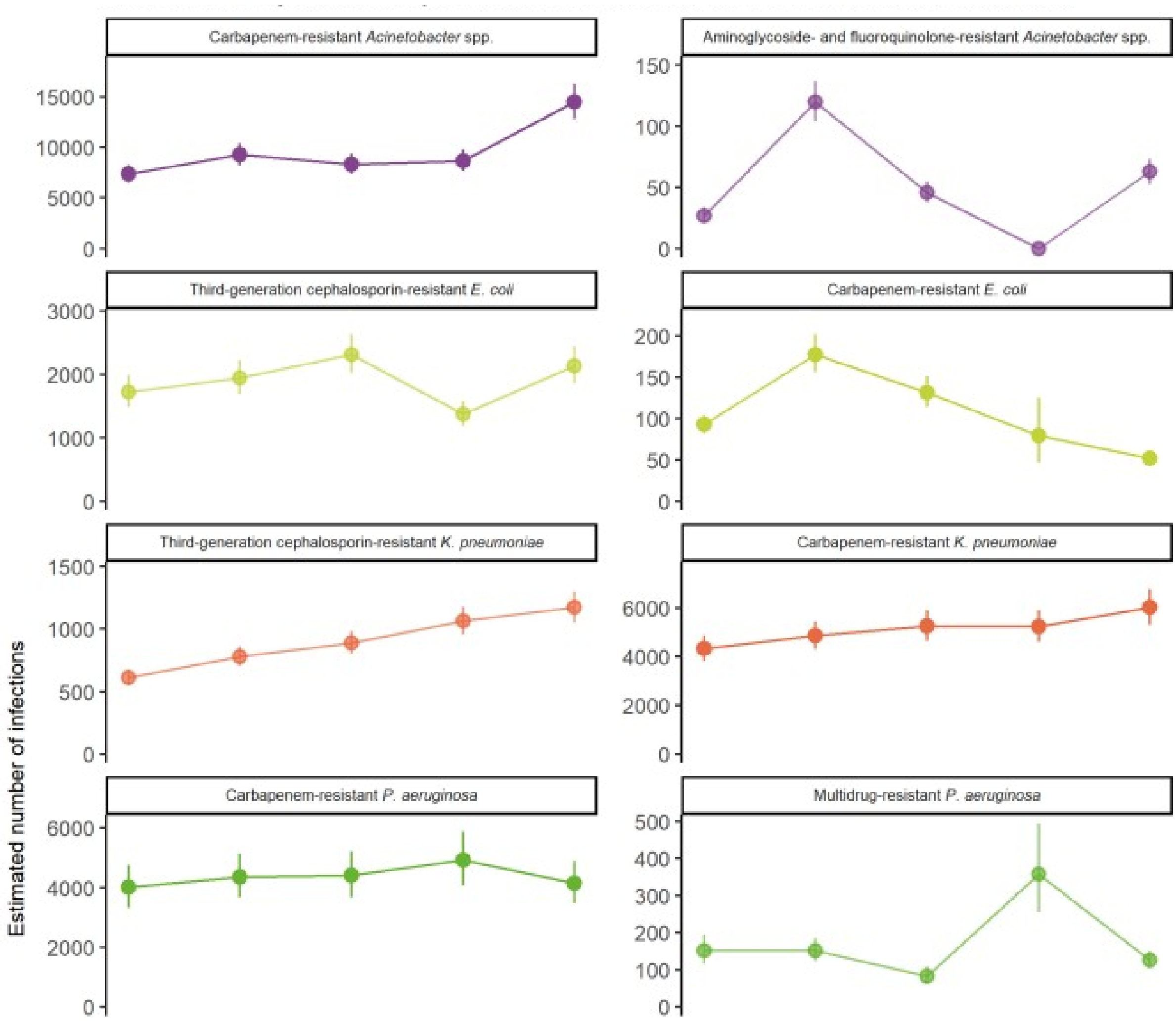


Figure 1.12 Greece: Estimated number of infections (bloodstream and other infections) with 95% uncertainty intervals, by bacterium- antibiotic resistance combination, 2016 – 2020 [66]

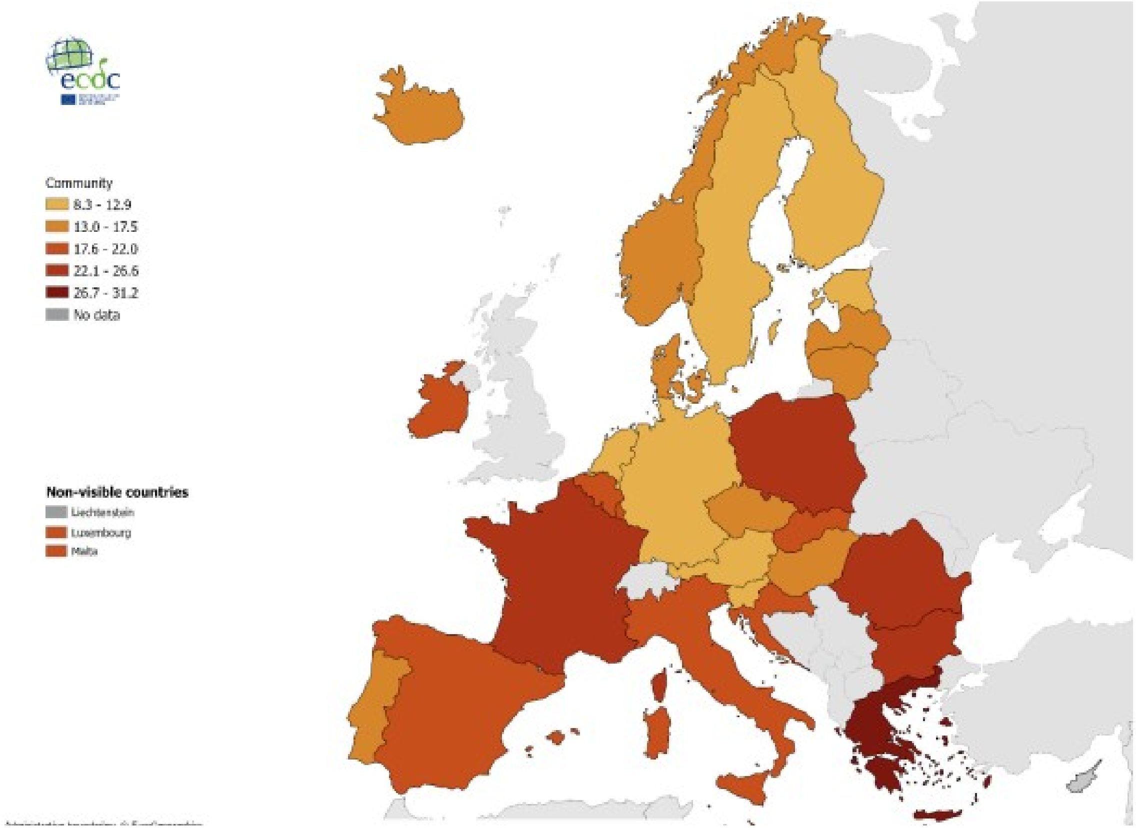


Figure 1.13 Community consumption of antibacterials for systemic use (ATC group J01), EU/EEA countries, 2022 (expressed as DDD per 1 000 inhabitants per day [67]

Country	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	Time series 2013–2022	Trend	Compound annual growth rate (CAGR)
Austria							34.0	34.8	36.5	35.3		N/A	N/A
Belgium	31.3	31.5	31.7	31.0	30.9	30.2	29.8	31.4	31.2	30.1		-	-0.5%
Bulgaria	52.7	57.5	55.2	59.8	58.1	57.5	58.5	62.6	71.1	67.5		↑	2.8%
Croatia	27.8	31.0	31.7	30.9	32.2	32.2	33.5	36.8	39.4	38.9		↑	3.8%
Cyprus												N/A	N/A
Czechia									23.5	15.9		N/A	N/A
Denmark	22.8	22.7	20.6	23.7	21.4	22.9	23.9	24.7	24.3	22.6		-	-0.1%
Estonia	19.3	20.0	20.0	20.6	23.2	19.8	21.8	24.6	24.8	23.2		↑	2.1%
Finland *(a)*	22.2	22.7	21.9	22.9	23.2	19.4	18.1	21.4	19.5	18.6		↓	-1.9%
France	31.3	32.3	32.2	31.3	31.6	32.6	30.1	32.4	33.4	36.3		-	1.7%
Germany												N/A	N/A
Greece	35.9	35.7	38.6	35.8	36.9	49.1	50.8	50.8	59.8	57.9		↑	5.4%
Hungary	37.8	37.4	38.8	39.4	40.1	40.6	36.3	40.8	42.4	39.7		-	0.6%
Iceland					16.4	18.1	17.0	18.7	21.3	29.3		N/A	N/A
Ireland	32.3	36.9	29.5	30.1	29.4	28.7	28.1	30.3	31.1	30.3		-	-0.7%
Italy	49.1	48.4	42.8	37.0	48.6	48.5	44.5	42.8	44.4	46.2		-	-0.7%
Latvia	40.1	39.9	40.1	39.9	38.4	38.1	40.8	36.9	41.9	40.2		-	0.0%
Lithuania	20.2	21.2	26.2	25.3	23.5	21.5	23.4	24.8	22.0	17.6		-	-1.5%
Luxembourg	31.1	31.8	33.3	36.0	34.0	35.7	35.1	37.9	37.5	35.1		N/A	1.4%
Malta	30.2	34.8	33.4	27.5	27.4	37.9	37.0	38.6	41.2	39.2		↑	2.9%
Netherlands	25.2	25.2	25.1	25.2	24.6	25.1	24.3	26.8	28.7	27.4		↑	0.9%
Norway	21.8	22.1	22.1	22.3	19.8	20.7	19.4	19.5	21.6	21.8		-	0.0%
Poland		23.6	24.1	34.2	24.3	31.8	29.2	34.3	44.1	37.9		N/A	6.1%
Portugal	42.6	43.8	43.6	43.5	42.2	42.8	42.6	43.3	44.2	42.6		-	0.0%
Romania							55.4	55.1	64.8	61.5		N/A	N/A
Slovakia	27.4	28.0	30.5	30.8	35.1	32.6	32.3	35.1	38.6	33.7		↑	2.3%
Slovenia	31.5	32.1	32.8	32.1	31.2	31.4	30.7	31.3	32.2	31.2		-	-0.1%
Spain				47.8	45.4	44.6	45.7	47.9	50.3	50.2		N/A	N/A
Sweden	25.2	27.1	26.5	27.4	26.9	24.5	27.9	28.6	30.4	30.0		↑	1.9%
EU/EEA*	**35.8**	**36.4**	**35.0**	**33.4**	**36.2**	**36.8**	**35.0**	**36.1**	**37.8**	**37.6**		-	**0.6%**
United Kingdom	*15.8*	*16.8*	*17.4*	*17.6*	*16.6*	*16.7*	*16.6*					*N/A*	*N/A*
*Reported EU/EEA***	*31.6*	*31.3*	*30.5*	*32.5*	*32.8*	*33.8*	*33.7*	*38.4*	*41.0*	*40.2*		*N/A*	*N/A*

Figure 1.14 Proportion (%) of glycopeptides, third- and fourth-generation cephalosporins, monobactams, carbapenems, fluoroquinolones, polymyxins, piperacillin and enzyme inhibitor, linezolid, tedizolid and daptomycin out of total hospital consumption (DDD per 1 000 inhabitants per day) of antibacterials for systemic use, EU/EEA and UK, 2013–2022 [67]

1.6 One Health approach on AMR surveillance

AMR is a global, multidimensional phenomenon occurring in humans, animals, and environmental ecosystems. The increasing emergence of AMR compromises our ability to treat infections and to manage AMR- associated economic impacts across all sectors [**53**]. Globalization, international travels and trade are some of the reasons which facilitate the rapid spread of AMR across borders and around the globe [**53, 54, 55**]. Therefore, it has become evident that tackling AMR effectively requires transnational and intercontinental partnerships [**53, 54, 55, 56**].

A unilateral approach to controlling AMR is insufficient. Preventing this silent pandemic requires multi-sectoral and transdisciplinary approach which is known as "One Health". One Health is a global strategy that recognizes the direct connection between human health and the health of animals and our shared environment. According to One Health approach, addressing severe public health issues can be achieved through cooperation, communication, and coordinated actions among professionals in human health (e.g., doctors, nurses, epidemiologists), animal health (e.g., veterinarians, agricultural workers), and environmental fields (e.g., ecologists, wildlife experts). [**53, 54, 55**].

The WHO recognizes the urgent need for coordinated global action to combat AMR and alongside the Food and Agriculture Organization (FAO) and the World Organisation for Animal Health (WOAH), coordinates global efforts, providing guidance, technical support, and monitoring progress [**53, 54, 57**].

Furthermore, in 2015 the WHO launched the Global Action Plan (GAP) which serves as a comprehensive framework for guiding efforts to address AMR issue. The GAP adopts the One Health approach and encourages countries to develop and implement National Action Plans (NAPs), and emphasizes the importance of multisectoral collaboration. Specifically, the GAP outlines five strategic objectives to address AMR at multiple levels [**53, 54, 55, 56**]:

A) Raise awareness and promote education among the general public, and healthcare professionals about the risks of AMR and on the appropriate use of antibiotics [**53-57**].

B) Develop and maintain robust AMR surveillance systems to track the spread of resistance. These surveillance systems include the environmental monitoring, such as tracking antibiotic residues and resistance gene prevalence and interventions to reduce contamination from agricultural runoff, healthcare waste, and industrial processes [**53, 54, 55**].

C) Implement effective infection prevention and control measures in healthcare settings, communities, and farms [**53, 54, 55**].

D) Enhance antimicrobial stewardship programs in order to regulate and monitor the use of antimicrobials in human medicine, veterinary medicine, and agriculture [**53, 54, 55**].

E) Encourage investments in new antimicrobials, diagnostics, and other tools to combat AMR [**53, 54, 55**].

1.7 AMR surveillance in Europe

The European strategy for combating AMR is firmly based One Health approach. The ECDC plays a central role in coordinating AMR surveillance across Europe. It manages several networks that collect and analyze data on resistant pathogens from clinical, veterinary, and environmental surveillance systems [**68, 69**].

The clinical surveillance of AMR is primarily concerned with monitoring resistance patterns in pathogens that infect humans and involves mandatory reporting of resistance data to databases, including:

1. The European Antimicrobial Resistance Surveillance Network (EARS-Net). EARS-Net is the largest AMR surveillance network in Europe, covering 30 countries [**68, 69**]. It monitors AMR in invasive bacterial pathogens from human clinical isolates, focusing on the ESKAPE pathogens (*E. faecium, Staph. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter species*), and especially on Carbapenem-resistant Enterobacteriaceae (CRE), Methicillin-resistant *Staph. aureus* (MRSA), Vancomycin- resistant *Enterococci* (VRE) and Extended-spectrum β-lactamases (ESBL)- producing *E. coli* [**68, 69, 70**].
2. Healthcare-Associated Infections Surveillance Network (HAI-Net): tracks infections acquired in healthcare settings, with a particular focus on those caused by resistant bacteria [**70**].
3. European Surveillance of Antimicrobial Consumption Network (ESAC-Net): monitors antibiotic consumption across Europe, providing data assist to assess the relationship between antibiotic use and the emergence of resistance [**71**].

Furthermore, Europe recognizing the importance of monitoring AMR in the environment (particularly in water bodies, soil, and wildlife) has developed:

1. NORMAN Network: a European initiative that monitors emerging environmental contaminants, including antibiotics and AMR genes. This network conducts joint monitoring campaigns, develops standardized methods for detecting AMR in the environment (such as water, soil and wastewater), and facilitates data sharing among European countries [**72**].
2. European Environment Agency (EEA): this supports the monitoring of environmental factors that contribute to AMR, such as antibiotic residues in water bodies and agricultural runoff [**73**].
3. Projects such as

Rethinking Antimicrobial Decision-systems in the Management of Animal Production (ROADMAP), which aims to optimize antimicrobial decision-making in animal production [**74**].

Joint Programming Initiative on Antimicrobial Resistance (JPIAMR), which funds research projects across Europe to explore AMR in the environment, including the impact of waste management practices and the role of wildlife in spreading resistance [**75**].

1.7.1 AMR surveillance in Greece

Greece's AMR surveillance system is coordinated by the National Public Health Organization (NPHO)and is supported by the WHOHET- Greece network and participates in both the EARS- Net (ECDC) and the Global Antimicrobial Surveillance System (GLASS- WHO) [**54, 68, 69**].

The Greek system places special emphasis on high-risk pathogens, including carbapenem- resistant gram negative bacteria, MRSA and VRE which are associated with high morbidity and mortality rates, particularly in hospitals settings. Given the critical situation of AMR, Greece has implemented the 'Procrustes' Action Plan since 2011, primarily targeting the control of CRE in hospitals. [**76**]

Now, Greece is actively working to enhance its surveillance systems and harmonize with international efforts to mitigate this threat. A National Action Plan on AMR "**National Action Plan for Combating Antimicrobial Resistance within the One Health Framework 2019-2023**" [**77**], which aligns with the WHO's Global Action Plan, has been developed [**54**]. This plan includes measures to improve antibiotic stewardship, to enhance surveillance and to strengthen infection prevention and control [**54, 61, 68, 69, 77**].

1.8 Characteristics of *E. coli*

E. coli is a Gram-negative, non- sporulating, rod- shaped, facultatively anaerobic coliform bacterium, which belongs to the Enterobacteriaceae family. It is motile due to peritrichous flagellar arrangement, and very few strains are non-motile. The optimal growth of *E. coli* occurs at 37°C, and under favorable conditions, it reproduces every 20 minutes [**20, 27**].

E. coli can live on a wide variety of environments. In general, it cycles between two major habitats:

a) the gastrointestinal tract of humans and other warm-blooded animals where it is a part of the microbiota. It synthesizes K and B complex vitamins protecting the host against colonization with pathogenics microbes [**20**].

b) environmental niches including water, wastewater, sediment, and soil, where it can live for long periods of time.

E. coli is used the most accurate indicator of fecal contamination, and in the domains of biotechnology and microbiology, it is the most widely studied prokaryotic model organism [**20, 27, 40**].

Although, more *E. coli* strains are harmless, certain strains are pathogenic causing intestinal or extraintestinal infections, depending on the array of virulence factors that they harbor. Various virulence factors, such as fimbrial and afimbrial adhesins, capsules, toxins (including exotoxins, hemolysins, and enterotoxins), and iron uptake systems, contribute to the pathogenicity of certain strains. [**20**].

The intestinal pathogenic *E. coli* (IPEC) strains are also linked to a number of extra-intestinal diseases and are the most prevalent cause of cholecystitis, bacteremia, cholangitis, urinary tract infections (UTIs), traveler's diarrhea, and septicemia as well as neonatal meningitis [**20**].

1.9 *E. coli* as an indicator of AMR in the environment

E. coli serves as a valuable indicator of AMR in the environment, given its ubiquitous widespread presence across various ecosystems, including the intestinal microbiota of mammals and birds [**20, 27, 40**]. Its ability to adapt genetically and its frequent exposure to antimicrobial agents make it a reliable marker for tracking AMR trends [**40, 78**]. Monitoring *E. coli* in environmental samples enables the assessment of resistance spread and dynamics, providing insights into the impact of antimicrobial use in both human and animal populations. This insight is instrumental in developing and refining policies to curb AMR spread [**40, 78**].

1.10 The One Health paradigm for AMR: extended-spectrum cephalosporin and carbapenem - resistant *E. coli*

While *E. coli* is intrinsically susceptible to nearly all clinically important antimicrobial agents, multidrug- resistant strains are frequently detected in both clinical and environmental samples. This is due to its remarkable ability to acquire resistance genes, primarily through horizontal gene transfer **[27, 40]**

The rise of multidrug resistance in *E. coli* has become a significant concern in both human and veterinary medicine globally. The most concerning resistance mechanisms in *E. coli* involve the acquisition of plasmid- borne genes that produce [**27, 40**]:

- Extended-spectrum β-lactamases (ESBLs) and AmpC enzymes confer resistance to a wide range of β-lactam antibiotics, including penicillins, broad spectrum cephalosporins, and aztreonam
- Carbapenemases causing resistance to carbapenems,
- Plasmid-mediated quinolone resistance (PMQR) genes, leading to resistance to quinolones (including fluoroquinolones)
- Plasmid- mediated genes that confer resistance to colistin.

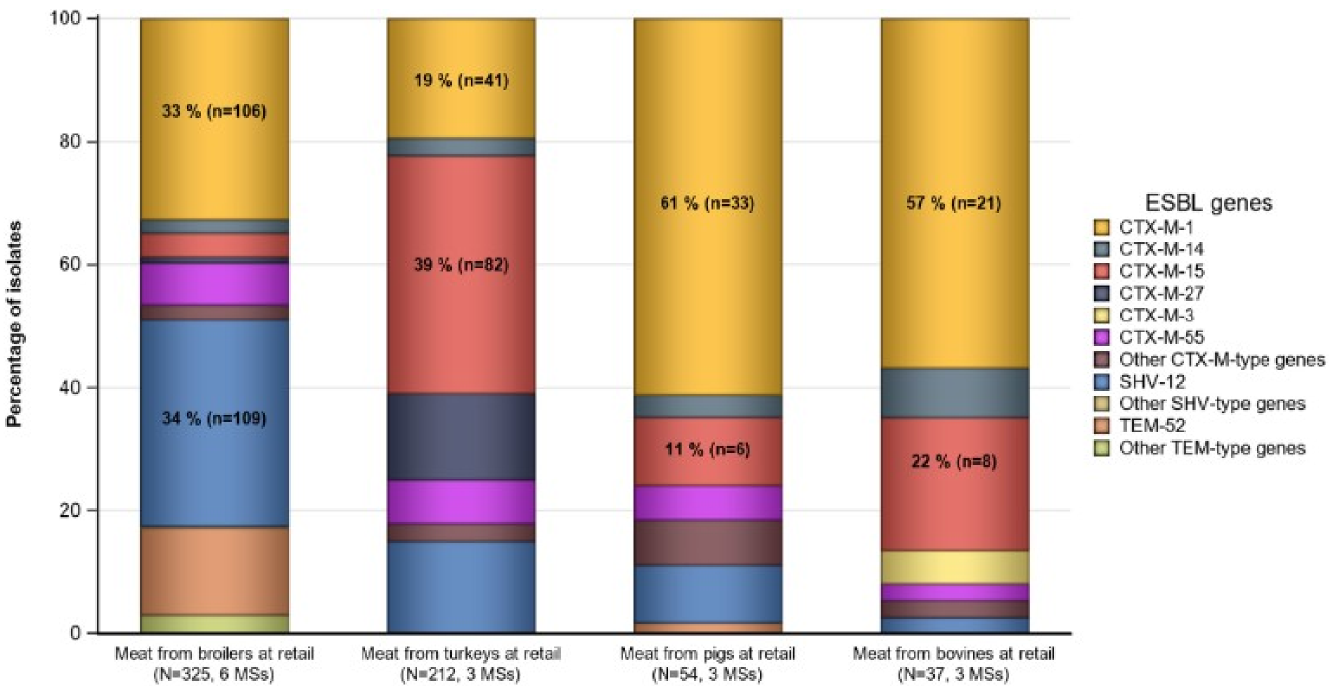
As mentioned before, infections caused by *E. coli* isolates producing ESBL and AmpC (referred to as extended-spectrum cephalosporin- resistant or ESC-EC) present a significant burden on healthcare systems. Furthermore, intestinal colonization by ESC-EC and its association with community-acquired multidrug- resistant (MDR) infections is a significant concern [**40, 78**]. Also, an increasing prevalence of ESBLs and AmpC genes has been observed in the human gut microbiota, affecting both healthy individuals and those with infections. Alongside this rising incidence in humans, ESC-EC are increasingly reported in livestock (**Figures 1.10a, b and 1.15a, c**), food products (**Figures 1.15b, d**), aquatic environments (**Figure 1.16**), and even wildlife (**Figure 1.17**) [**40, 78, 79**]. The most widely reported cephalosporinases in *E. coli* from humans are CTX-M-1, followed by CTX-M-15, CTX-M-14, and CMY-2 [**27, 40, 78, 79**]. According to epidemiological data from EFSA and other studies, blaCTX-M-1 and blaCTX-M-15 are the most prevalent ESBL genes found in both livestock and wild animals, while the most widespread AmpC-encoding gene is blaCMY-2. Additionally, blaCTX-M-15 is also predominant in aquatic ecosystems [**78, 79**].

In addition to extended-spectrum cephalosporin- resistant *E. coli* (ESC-EC), the rising prevalence of carbapenemase- producing *E. coli* (CP-EC) strains poses a significant concern. Research has confirmed the presence of CP-EC in food- producing animals, animal- derived food products, companion animals, and aquatic environments [**40, 61, 63, 78, 79**]. Among CP-EC, the blaOXA-181 gene is the most frequently identified carbapenemase gene, followed by blaNDM-5 [**63, 80, 81**]. While blaOXA-181 is predominantly associated with human infections, it has also been detected in various environmental sources such as seawater and hospital sewage. Additionally, blaOXA-48-like variants, including blaOXA-204 and blaOXA-244, have been found in river water, estuaries, and alarmingly, in drinking water [**63, 80, 81**].

The blaNDM-5 gene, the second most common carbapenemase gene in CP-EC, has been isolated from companion and food-producing animals [**63, 80, 81**]. Notably, blaNDM-5 is the most frequently reported carbapenemase gene in CP-EC among humans in Europe [**61, 82**]. Also, blaNDM genes have been detected in *E. coli* isolated from municipal and hospital sewage, rivers, and, more worryingly, drinking water [**80, 81**]

CP-producing Enterobacterales have been identified not only in EU monitoring programs [**61, 82, 83, 84, 85**] but also in companion animals **[81]**, food-producing animals, and their derived products such as meat, seafood, and vegetables [**80, 81, 83, 86, 87, 88, 89**]. The amplification of carbapenemase- producing *E. coli* in foods, which are considered a significant reservoir of these bacteria for humans, is highly undesirable. The global spread of ESC-EC and CP-EC underscores the urgent need for comprehensive surveillance and targeted intervention strategies to mitigate this escalating public health threat [**83, 84, 86, 88, 89**]. The increasing prevalence of these resistant bacteria across diverse environments and hosts highlights the complexity of controlling their dissemination [**88, 89, 90**]. Ongoing efforts to develop scientifically-based intervention measures are crucial to addressing this growing public health issue [**40, 62, 80, 86, 90**].

a)



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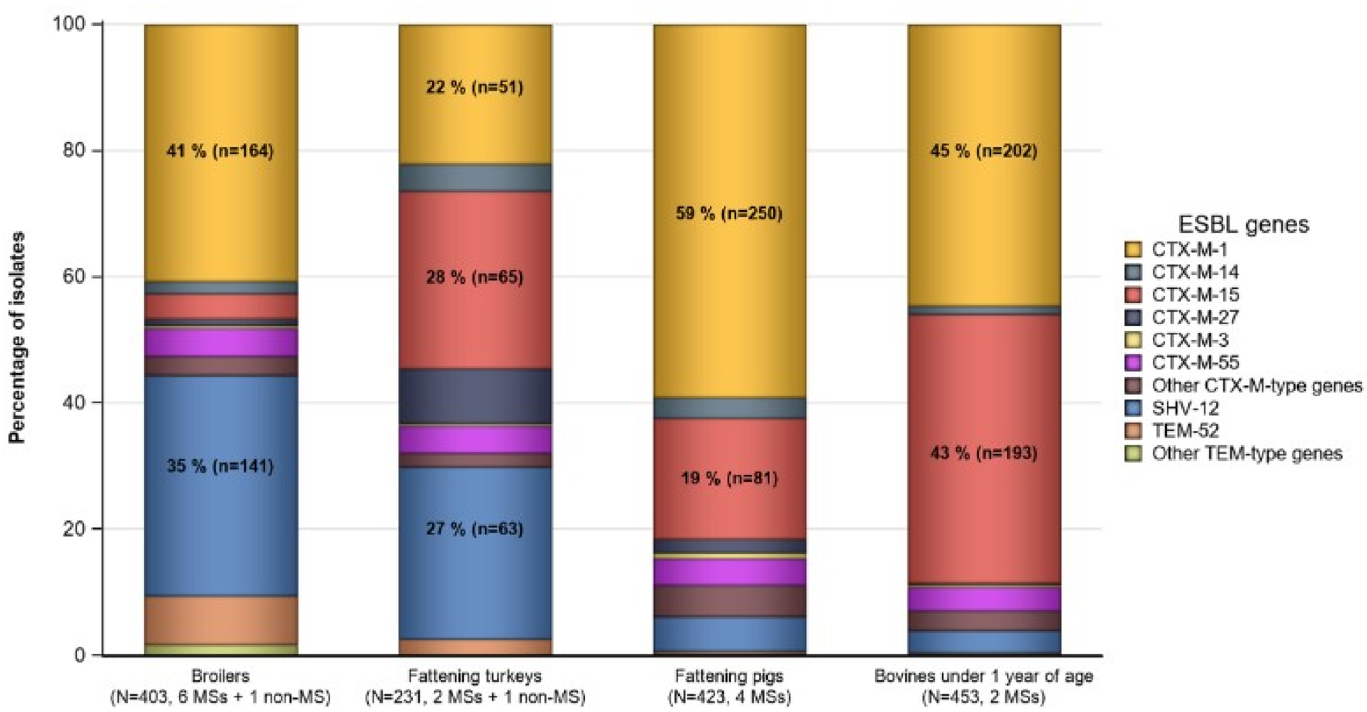
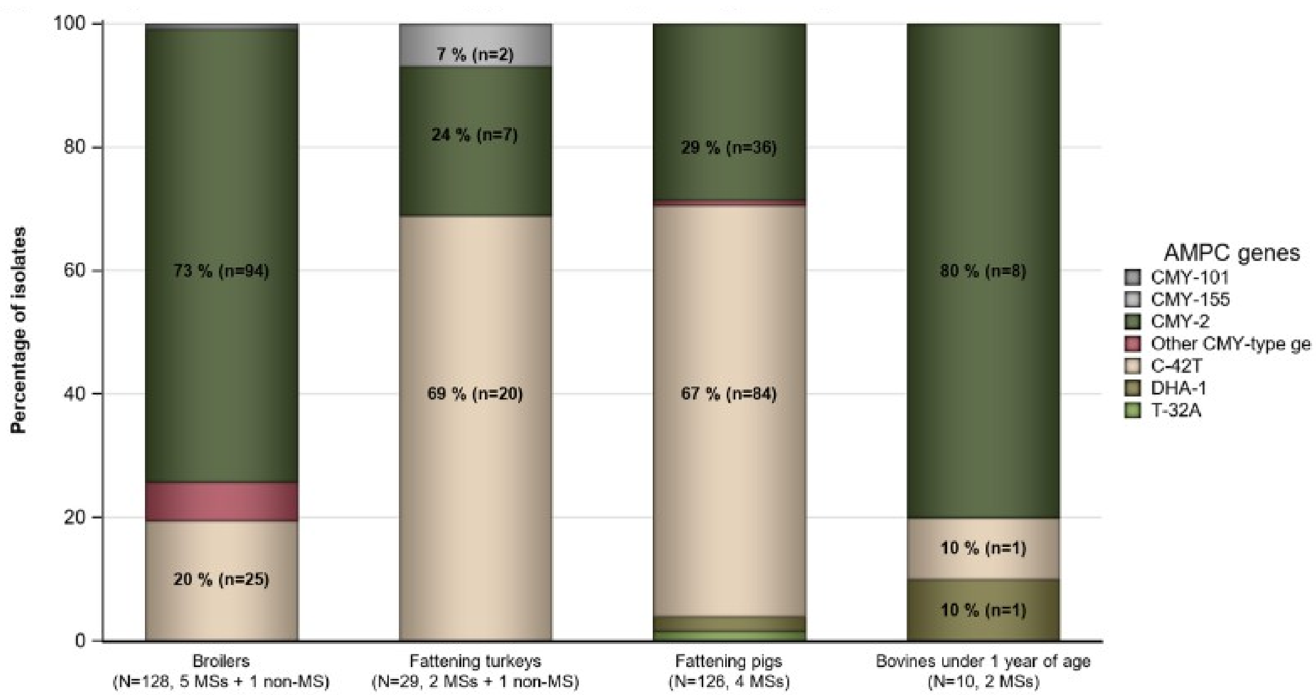


Percentage of isolates
100
80
60
40
20
0
41 % (n=164)
35 % (n=141)
22 % (n=51)
28 % (n=65)
27 % (n=63)
59 % (n=250)
19 % (n=81)
45 % (n=202)
43 % (n=193)
Broilers
(N=403, 6 MSs + 1 non-MS)
Fattening turkeys
(N=231, 2 MSs + 1 non-MS)
Fattening pigs
(N=423, 4 MSs)
Bovines under 1 year of age
(N=453, 2 MSs)
ESBL genes
CTX-M-1
CTX-M-14
CTX-M-15
CTX-M-27
CTX-M-3
CTX-M-55
Other CTX-M-type genes
SHV-12
TEM-52
Other TEM-type genes

c)



Percentage of isolates
100
80
60
40
20
0
73 % (n=94)
20 % (n=25)
7 % (n=2)
24 % (n=7)
69 % (n=20)
29 % (n=36)
67 % (n=84)
80 % (n=8)
10 % (n=1)
10 % (n=1)
Broilers
(N=128, 5 MSs + 1 non-MS)
Fattening turkeys
(N=29, 2 MSs + 1 non-MS)
Fattening pigs
(N=126, 4 MSs)
Bovines under 1 year of age
(N=10, 2 MSs)
AMPC genes
CMY-101
CMY-155
CMY-2
Other CMY-type ge
C-42T
DHA-1
T-32A

d)

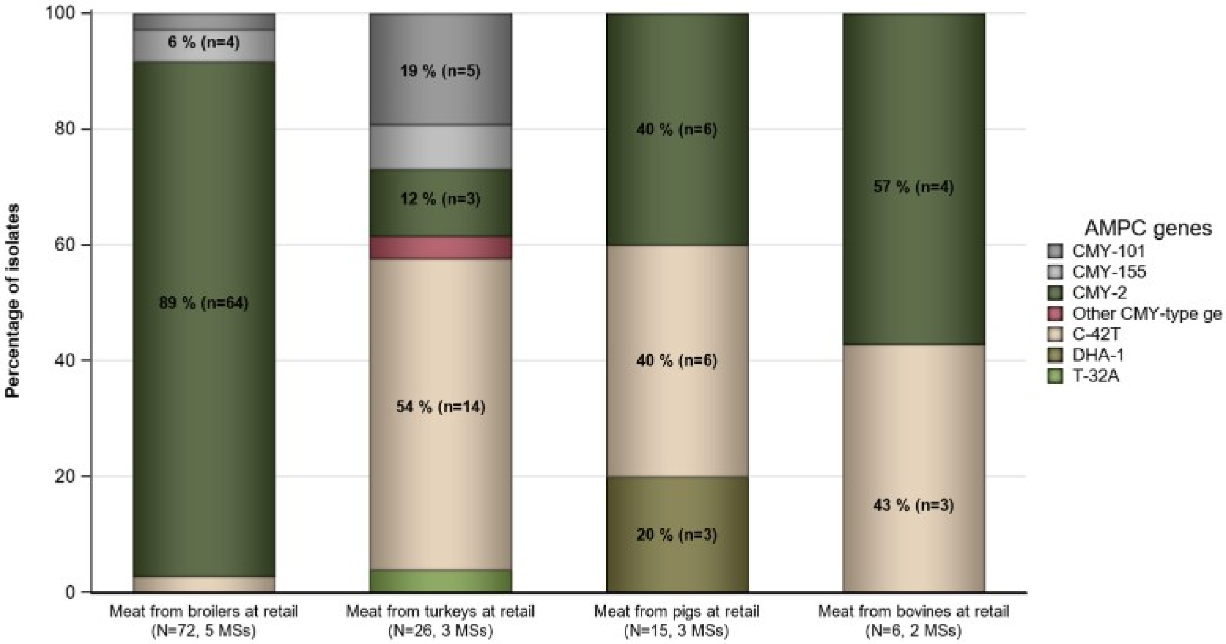


Figure 1.15 E. coli* isolates *harboring (a) ESBL- encoding genes in animals, (b) ESBL- encoding genes in retail meat, (c) AmpC- encoding genes and AmpC- chromosomal point mutations in animals and (d) AmpC- encoding genes and AmpC- chromosomal point mutations in retail meat. EFSA 2024 [63]

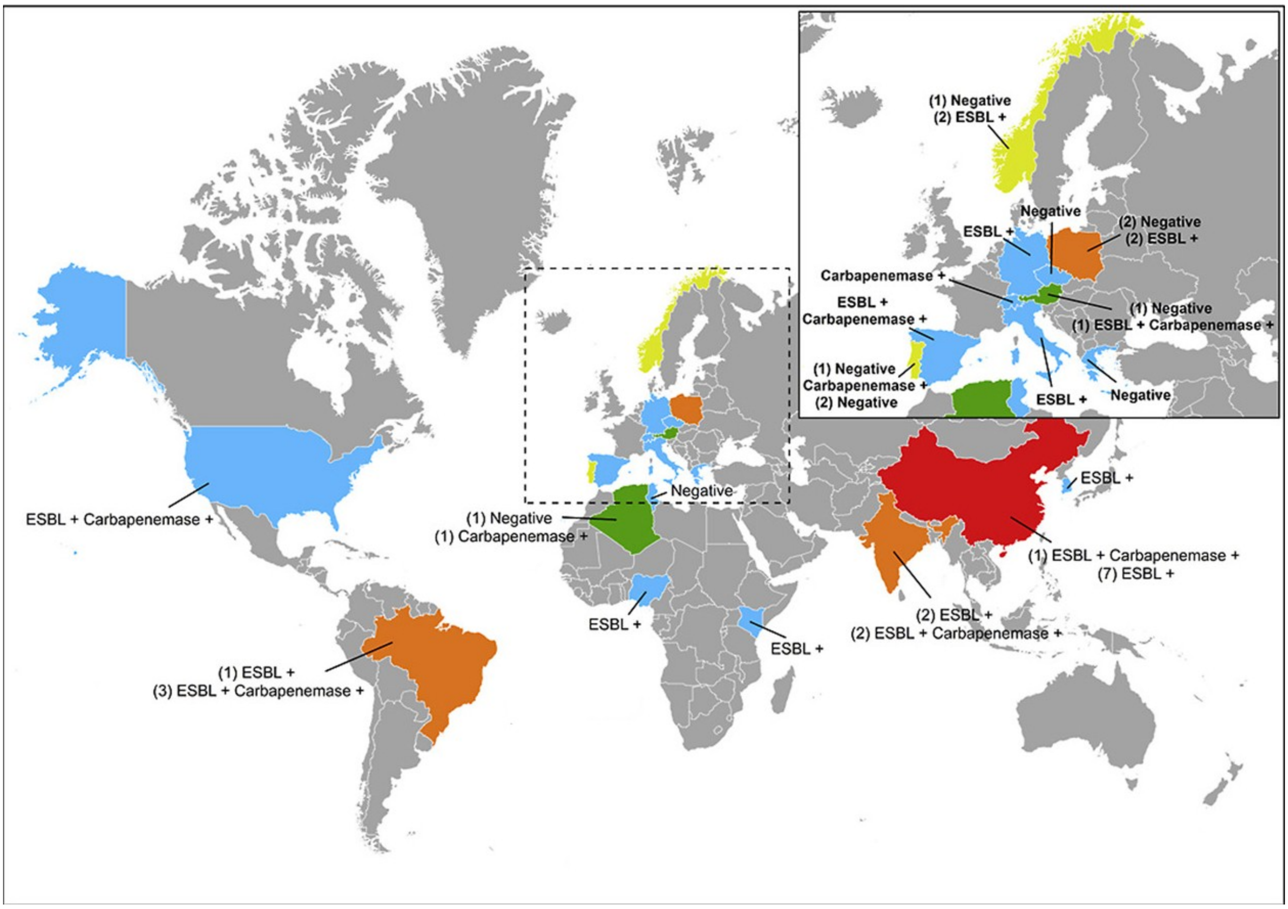


Figure 1.16 Global distribution of positive detection of ESBL and/or carbapenemase genes in aquatic environments such as rivers, lakes and ground water [89].

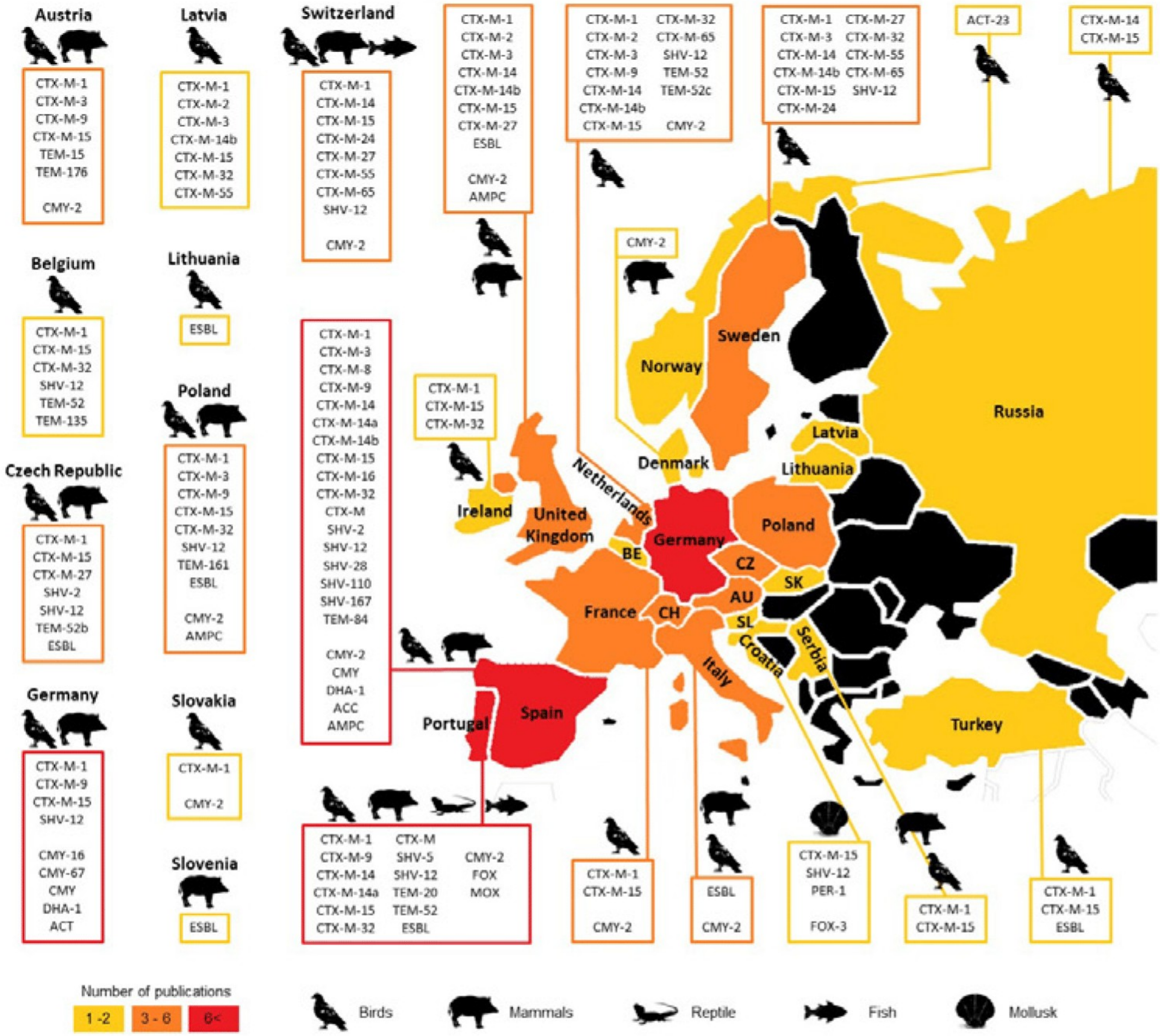


Figure 1.17 Map of Europe showing the animal host and cephalosporinases subtypes [90]

Chapter 2

Experimental Part

Material & Methods

Objective

This doctoral dissertation was conducted as part of the One Health approach. Specifically, the primary purpose of this dissertation was to assess the antimicrobial resistance patterns, the genes related to specific resistant phenotypes, and molecular genotypes by means of phylogenetic groups of *E. coli* isolates circulating both in environmental habitats (including sewage and receiving water bodies) and clinical settings.

Another key objective of this dissertation was the epidemiological correlation of genotypes circulating in different environmental settings with those predominant in hospitals, as well as the correlation between genotypes and resistance profiles.

Finally, a subset of the isolated strains that met specific criteria underwent plasmid sequencing using modern sequencing techniques to reveal molecular mechanisms, such as the presence of mobile elements (integrons, transposons), related to the spread of antimicrobial resistance in these environments.

Ethics Statement

This study has received approval from the Bioethics and Research Ethics Committee of the University of West Attica (Reference Number: 33114/13-04-2021).

2.1 Sampling locations and collected samples

Sampling locations and collection procedures were strategically chosen to capture the distribution of environmental AMR in Livadeia city (the capital of the regional unit) of the Boeotia regional district, Greece. This region was chosen due to its intensive agricultural and farming activities, and it is crossed by two rivers: the Erkyna River on the northern side of the city and the Boeotian River on the southeast side of the city. The area also hosts a WWTP and a general prefectural hospital which performs a semi-treatment on the HWW. The hospital provides a wide range of services to approximately 60,000 people annually, including emergency and outpatient care, and has clinics for nephrology, pathology, cardiology, surgery, orthopedics and obstetrics–gynecology. After preliminary sedimentation, the hospital sewage is discharged into the regional WWTP. The WWTP receives urban and HWW, with an average daily volume of 5500 m3/day at the entrance and an average hourly flow of 400 m3/h. It performs primary treatment, including screening, grit collection, grease trap, oxidation ditch and primary sedimentation, as well as biological treatment that includes nitrogen and phosphorus removal, secondary sedimentation, chlorination, sludge thickening and dewatering [**15**]. The secondary treated effluents are disposed of in the Erkyna river, and are used for the restricted irrigation of cropland during the irrigation season. The Erkyna river is directly influenced by the WWTP discharges and indirectly by the hospital sewage. The Erkyna river flows into the Boeotian Kifissos river at a point approximately 6 km away from the WWTP. Both rivers are used for irrigation purposes, with one irrigation project covering 16,000 acres of the studied area [**91**].

During the period of summer 2019 to spring 2021, six sequential sampling events were conducted in Livadeia city, Boeotia regional district. A total of four samples per sampling period were collected, including (a) semi-treated HWW from a septic tank outside the hospital, (b) wastewater at the outlet of the regional WWTP, (c) river water samples from the Erkyna river adjacent to the WWTP (RWS1) and (d) river water samples from the Boeotian Kifissos river at the junction with the Erkyna river (RWS2), located 6 km downstream from the WWTP (**Figure 2.1**). A total of twelve river water samples (six from RWS1 and six from RWS2), six wastewater samples and six HWW samples were collected and analyzed. All of the samples were collected in sterile dark bottles (500 mL volume), were placed on ice and analyzed within 12 h post-collection. In addition, clinical isolates were collected from clinical specimens such as urine, blood and tissue from the microbiological laboratory of the hospital during the whole study period.

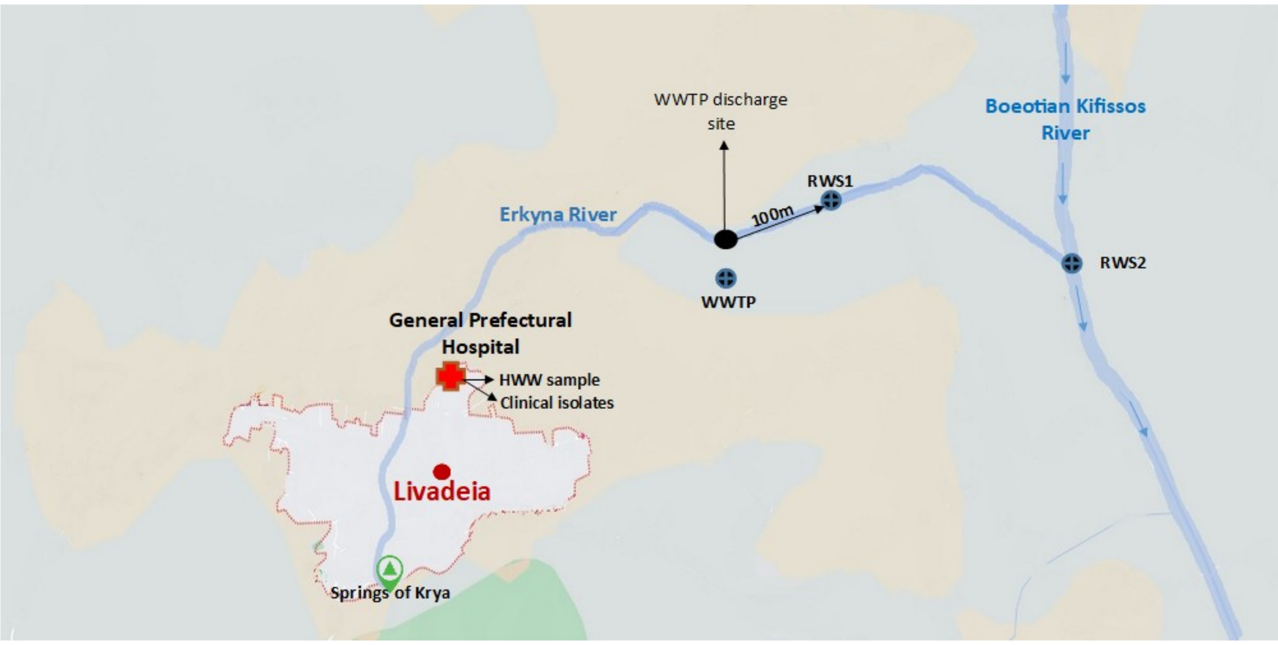


Figure 2.1 The map depicts the sampling locations and their relationships.

[The abbreviations used in the map are as follows: HWW, hospital wastewater; WWTP, wastewater treatment plant; RWS1, river water site 1 (located 100m downstream from the WWTP discharge site); RWS2, river water site 2 (located 6 km downstream from the WWTP discharge site].

2.2 Isolation of environmental *E. coli* strains

Materials and equipment

- Culture Media: Chromogenic Coliform Agar, CCA (CHROMagar™ CCA, EF342)
- Cellulose ester membrane (Whatman® ME 25/21 ST)
- Antibiotics: Ampicillin Sodium for Injection 1 Gram (AUROMEDICS, NDC 55150-0113-10)
- Multiple vacuum filtration device (Whatman®, AS310/3, WHA10445835)

Method

E. coli isolation and identification were conducted using a standard membrane filtration technique [***ISO 9308.01-1: 2017,* 92**] for all river and wastewater samples. The procedure involved filtering multiple volumes (river water: 100 mL, 10 mL, 1 mL, wastewater: 10 mL, 1 mL, 0.1 mL) of each sample using a vacuum filtration device and a mixed cellulose ester membrane with a diameter of 47 mm and pore size of 0.45 μm (Whatman® ME 25/21 ST). The membrane filters were then placed sterile petri dishes with Chromogenic Coliform medium (CCA) with and without an antibiotic (CCA with 100 μg/mL ampicillin, CCA/AMP). In both culture media with and without AMP, all colonies showing positive β-d-galactosidase and β-d-glucuronidase reactions (dark blue to violet) were counted as *E. coli*. The CCA/AMP was used for the estimation and collection of the β-lactam-resistant isolates, while CCA without AMP was used for the enumeration and isolation of all *E. coli* isolates (e.g., sensitive and resistant to all antibiotics).

All suspected *E. coli* (dark blue to violet) colonies which were isolated from CCA and CCA/AMP were subcultured on Mac Conkey No3 agar, a selective and differential culture medium. Mac Conkey No3 is designed to selectively isolate only Gram-negative bacteria and differentiate them based on lactose fermentation. The pink /red metallic sheen appearance of *E. coli* colonies on MacConkey agar No3 agar was used to identify their features [**92**].

In cases of doubt concerning the *E. coli* colonies, additional biochemical and molecular identification tests were applied. For biochemical testing, all isolated colonies were maintained on a nutrient agar [**92**].

2.3 Biochemical identification of environmental *E. coli* strains

Materials

- MacConkey Agar No.3 (Neogen® Culture Media, NCM0174B)
- Nutrient Agar (Neogen® Culture Media, NCM0033A)
- Peptone Water/ Tryptone Water (Condalab, Cat. 1403)
- Kovac's Reagent (Liofilchem, 80271)
- Simmons Citrate Agar (Neogen® Culture Media, NCM0168A)

Methods

E. coli colonies on MacConkey agar No3 agar with pink /red metallic sheen appearance subcultered on Nutrient agar and after overnight incubation at 37oC, they were subjected to indole and Simmons citrate biochemical tests.

2.3.1 Indole biochemical test

The indole test screens for the ability of a bacterium to degrade the amino acid tryptophan and produce indole. It is used as a classic test to distinguish indole-positive *E. coli* from indole negative Enterobacteriaceae such as Klebsiella [**92, 93**]. For this test, a single colony of a pure culture was inoculated in a tube with tryptone/peptone broth and after overnight incubation at 37°C, five drops of Kovács reagent were added directly to the tube in order to test indole production [**92, 93**]. A positive indole test is indicated by the formation of a red color ("cherry-red ring") in the reagent layer on top of the medium within few seconds [**92, 93**].

2.3.1 Simmons citrate biochemical test

The citrate test screens a bacterial isolate for the ability to utilize citrate as its carbon and energy source. This test is employed in combination with the indole test to distinguish between members of the Enterobacteriaceae [**92, 94**]. To carry out the test, a single colony of a pure culture was inoculated on Simmons media followed by incubation at 37°C for 18 to 48 hours. Only bacteria that can utilize citrate as the sole carbon will be able to grow on the Simmons citrate medium and the generation of alkaline by-products of citrate metabolism raise the pH of the medium causing the bromothymol blue to change from the original green color to blue. In cases of bacteria such as *E. coli* that give negative citrate test, no growth and no color change will be visible in the media [**94**].

2.4 Isolation and identification of clinical *E. coli* strains

As for the clinical strains, they were obtained from biological fluids of hospitalized or emergency room patients, such as blood, urine and tissue, and were identified as *E. coli* in the microbiological laboratory of the hospital. Specifically, the clinical samples were cultivated on blood and MacConkey agar No3 at 37 °C for 24 h. Following this, the isolates were identified via a Micro Scan automated system according to standard biochemical tests. The isolates were stored in cryovials with Brain Heart Infusion+20% glycerol solution and transported to the Molecular Microbiology and Immunology Laboratory with proper packaging and transfer conditions [**95**].

2.5 Molecular identification of *E. coli* isolates

Material and equipment for DNA extraction and PCR

- Nutrient Agar (Neogen® Culture Media, NCM0033A)
- Water for injection
- DreamTaq DNA Polymerase, 5 U/μL (Thermo Scientific™, EP0702)
- 10X DreamTaq Buffer (includes 20 mM MgCl2) (Thermo Scientific™, EP0702)
- Primers of 100 μM stock solution, for housekeeping uidA gene (Invitrogen- Thermo Fisher Scientific)
- Deoxynucleotide triphosphates (dNTPs) Bundle, 4x 100 mM (dATP, dCTP, dGTP, dTTP), (JENA BIOSCIENCE, NU-1005S)
- *Escherichia coli* ATCC 25922 (positive control isolate)
- Mini Centrifuge (MiniSpin ® Eppendorf, 22331)
- PCR Thermocycler 2720 (Applied Biosyststems, Thermo Fisher Scientific)

Materials and equipment for electrophoresis

- FastGene Agaroze (Nippon Genetics, Cat.:AG02)
- Midori Green Advance gel stain (Nippon Genetics Cat. No.: MG04)
- 10X Tris – Borate EDTA (TBE) stock solution: 900 mM Tris base, 900mM Boric acid and 20mM EDTA pH 8.0
- BlueJuice™ Gel Loading Buffer 10x (Invitrogen™ by Thermo Fisher Scientific, Cat. No: 10816015)
- DNA ladder (FastGene 100 bp DNA Marker, Nippon Genetics Cat. No. MWD100)
- Horizontal electrophoresis tank ClearSub L10 (Kisker Biotech)
- Gel imaging system FastGene FAS-DIGI PRO (Nippon Genetics)

Method

The final confirmation of isolates identity was achieved using the molecular method of polymerase chain reaction (PCR) targeting the housekeeping β-d-glucuronidase gene uidA [**95**]. Genomic DNA extracted by the boiling method as follow: few colonies of fresh pure cultures grown on Nutrient agar were resuspended in 250 µL water for injection, lysed by heating at 100 oC for 25 min and then were immediately put on ice for 10 min. The supernatant, which contains the whole bacterial genome, was harvested by centrifugation at 11.000 rpm for 10 min. Then, the bacterial genomic DNA was amplified by PCR using a specific set of primer targeted at uidA gene. The pair of primer used for uidA PCR amplification is shown in (**Table 2.1**). Each reaction was carried out by using a 25 µl mixture containing 2.5µl of 10× DreamTaq buffer (includes 20mM MgCL2), 0.5 µM of each primer (initial concentration 10 µM), 0.2 mM of each dNTP (initial concentration of dNTPS mix 10 mM), 1 U of DreamTaq polymerase, and 3 µl of genomic DNA. The PCR was performed under the following conditions: denaturation for 6 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 56°C and 1.5 min at 72°C; and a final extension step of 10 min at 72°C [**96**]. Negative control (reaction lacking the template DNA) and a positive control (*Escherichia coli* ATCC 25922) were included in all performed amplifications. Six-microliter aliquots of PCR products were analyzed by gel electrophoresis with 1.5 % agarose in 1X TBE. Gels were stained with Midori Green stain and visualized in Gel imaging system FastGene FAS-DIGI PRO. A 100-bp DNA ladder was used as a marker.

Table 2.1 The pair of primer used for uidA PCR amplification [96]

Target gene	**Sequences 5’→ 3’**	**Melting temperature-Tm (oC)**	**Product size (bp)**
uidA	**F_GTTTTCCCAGTCACGACGTTGTACATTACGGCAAAGTGTGGGTCAAT** **R_TTGTGAGCGGATAACAATTTCCCATCAGCACGTTATCGAATCCTT**	**56**	**740**

2.6 Storage of isolates

Materials

- Nutrient Agar (Neogen® Culture Media, NCM0033A)
- Brain Heart Infusion Broth (Thermo Scientific™ CM1135B)
- glycerol
- cryovials

Method

All isolates that exhibited positive and negative result in indole production and citrate test, respectively and simultaneously indicated positive result in uidA pcr, were presumed to be *E. coli* and were stored as stock at -80 ºC in cryovials with Brain Heart Infusion and 20% glycerol solution, for further experiments.

2.7 Antimicrobial susceptibility testing and phenotypic methods for detecting antibacterial resistance mechanisms

Materials

- Petri dishes 120x120 mm
- Muller Hinton Agar I ((Neogen® Culture Media, NCM2016A)
- 0,9% NaCl
- Antibiotic disks (LIOFILCHEM ®, ITALY and BIOPROM BD, Greece)
- *Escherichia coli* ATCC 25922 (reference strain for antimicrobial susceptibility testing and other phenotypic tests)

Methods

2.7.1 Antimicrobial susceptibility testing

All isolates (environmental and clinical) were tested for their antimicrobial susceptibility via disk diffusion assays (Kirby–Bauer method) in 18 antibiotics, commonly used in clinical practice, distributed in 9 different categories: penicillins (ampicillin (AMP; 10 μg), piperacillin (PIP; 30 μg)), penicillin/inhibitor combinations (amoxicillin/clavulanic acid (AMC; 20 μg/10 μg), piperacillin/tazobactam (TZP; 30 μg/6 μg)), cephalosporins (ceftriaxone (CRO; 30 μg), cefuroxime (CXM; 30 μg), ceftazidime (CAZ; 10 μg), cefotaxime (CTX; 5 μg), cefepime (FEP; 30 μg)), cephamycins (cefoxitin (FOX; 30 μg)), monobactams (aztreonam (ATM; 30 μg)), carbapenems (imipenem (IMP; 10 μg), meropenem (MEM; 10 μg)), aminoglycosides (amikacin (AN; 30 μg), gentamicin (GM; 10 μg)), quinolones (ciprofloxacin (CIP; 5 μg), nalidixic acid (NAL; 30 μg)) and miscellaneous agents (sulfamethoxazole-trimethoprim (SXT; 23.75 μg/1.25 μg)). The test was performed by inoculating a bacterial suspension (of turbidity equal to 0.5 of the McFarland scale) onto Muller Hinton agar followed by placing antibiotic-impregnated paper disks on the surface. Antibiotic disks were positioned at a distance of 30 mm (centre to centre).The interpretation of the susceptibility results for the environmental and clinical isolates was performed according to EUCAST ECOFFs (epidemiological cut-off values) and clinical breakpoint criteria, respectively [**97**]. All isolates were characterized as sensitive/wild-type (S/WT: susceptible to all antibiotics), as non-wild-type (N-WT: resistant to only one antibiotic factor), as resistant (R: resistant to more than one antimicrobial agent; maximum of three different categories) or as multi-drug-resistant (MDR: resistant to at least one antimicrobial agent in more than three categories) [**97, 98, 99**].

2.7.2 Phenotypic methods for detecting antibacterial resistance mechanisms

2.7.2a: Double-disk synergy test for the detection of extended spectrum β-lactamases (ESBL) production

Clavulanic acid synergy test (double-disk synergy test, DDST) is recommended for the detection of extended spectrum β-lactamases (ESBL) production in isolates with resistance to third and/or four - generation cephalosporins (such as cefotaxime, ceftazidime, cefepime) [**100**]. The test was performed by inoculating a bacterial suspension (of turbidity equal to 0.5 of the McFarland scale) onto Muller Hinton agar followed by placing disks containing cephalosporins (cefotaxime, ceftazidime, cefepime) next to a disk with clavulanic acid (amoxicillin-clavulanic acid, AMC). The test was considered positive when the inhibition zones around any of the cephalosporin disks were augmented in the direction of the disk containing clavulanic acid [**100**].

2.7.2b Carbapenem inactivation method for detection of carbapenemase production

The phenotypic test carbapenem inactivation method (CIM) were implemented in isolates which exhibited decreased susceptibility to carbapenems (meropenem, imipenem) in order to detect carbapenemase production such as KPC, NDM, OXA-48, VIM, IMP and OXA-23 [**100, 101**]. The CIM test detects carbapenemase production by incubating a bacterial suspension with a carbapenem disk (e.g meropenem). If carbapenemase is present, it hydrolyzes and inactivates the drug. After incubation (typically around 2 hours), the disk is placed on Muller Hinton agar inoculated with susceptible *E. coli* strain (commonly ATCC 25922). A reduced or absent inhibition zone around the disk indicates carbapenemase production, while a clear inhibition zone suggests no enzyme activity

[**101**]. Well-characterized carbapenem- producing bacterial strains, provided by the Laboratory of Antimicrobial Resistance and Hospital Infections, National Reference Center for Infectious Diseases, Hellenic National Public Health Organization, were used as positive controls for the CIM test.

2.8 PCR amplification of resistance genes

Materials and Equipment for DNA extraction and PCR

- Nutrient Agar (Neogen® Culture Media, NCM0033A)
- Water for injection
- Mini Centrifuge (MiniSpin ® Eppendorf, 22331)
- PurelinkTM Genomic DNA mini kit (Invitrogen, Waltham, MA, USA)
- DreamTaq DNA Polymerase, 5 U/μL (Thermo Scientific™, EP0702)
- 10X DreamTaq Buffer (includes 20 mM MgCl2) (Thermo Scientific™, EP0702)
- Primers of 100 μM stock solution, for housekeeping uidA gene (Invitrogen- Thermo Fisher Scientific)
- Deoxynucleotide triphosphates (dNTPs) Bundle, 4x 100 mM (dATP, dCTP, dGTP, dTTP), (JENA BIOSCIENCE, NU-1005S)
- PCR Thermocycler 2720 (Applied Biosyststems, Thermo Fisher Scientific)
- Positive controls- well characterised strains positive for the tested resistance genes.
- NucleoSpin, Gel and PCR clean-up (MACHEREY-NAGEL)

Materials and equipment for electrophoresis

- FastGene Agaroze (Nippon Genetics, Cat.:AG02) for gel electrophoresis
- Midori Green Advance gels stain (Nippon Genetics Cat. No.: MG04)
- 10X Tris – Borate EDTA (TBE) stock solution: 900 mM Tris base, 900mM Boric acid and 20mM EDTA pH 8.0
- BlueJuice™ Gel Loading Buffer 10x (Invitrogen™ by Thermo Fisher Scientific, Cat. No: 10816015)
- DNA ladder (FastGene 100 bp DNA Marker, Nippon Genetics Cat. No. MWD100)
- Horizontal electrophoresis tank ClearSub L10 (Kisker Biotech)
- Gel imaging system FastGene FAS-DIGI PRO (Nippon Genetics)

Method

All DDST-positive isolates underwent PCR to detect three different types of ESBL genes: blaTEM, blaSHV and blaCTX-M-group 1-, 2-, 9- types [**102, 103**]. CIM-positive isolates were tested for the presence of carbapenemase genes (blaKPC, blaVIM, blaNDM, blaIMP, blaOXA-48 and blaOXA-23) [**100, 102, 104**]. Isolates resistant to penicillin/inhibitor combinations and cephamycins were tested for AmpC-type β-lactamases genes (blaCMY, blaDHA, blaACC, blaMIR and blaFOX) [**100, 105**], while MDR isolates exhibiting resistance to SXT were screened for the dihydropteroate synthase gene (sul1) demonstrating resistance to sulphonamides [**106**]. PCR protocols and conditions were performed according to EUCAST guidelines and other published studies [**99, 100-105**].

The total bacterial genome of all the above isolates was extracted using the Purelink™ Genomic DNA Mini Kit according to the manufacturer's instructions. Each reaction was carried out by using a 25 μl mixture containing 2.5 μl of 10× Dream Taq Buffer (+MgCl2), 0.5 μM of each primer (initial concentration 10 μM), 0.2 mM of each dNTP (initial concentration of dNTPS mix 10 mM), 1 U of DreamTaq polymerase, and 100 ng of bacterial genomic DNA. PCR conditions for the amplification of ESBL genes are displayed in **Table 2.2**, of AmpC genes in **Table 2.3**, carbapenemase genes in **Table 2.4** and sul1 gene in **Table 2.5**. All primer sets used for resistance genes detection are listed in **Table 2.6**. Negative control (reaction lacking the template DNA) and positive controls were included in all performed amplifications. All bacterial strains used as positive controls were kindly provided by the Laboratory of Antimicrobial Resistance and Hospital Infections, National Reference Center for Infectious Diseases, and Hellenic National Public Health Organization. These strains either originate from inter-laboratory schemes in which the laboratory participates or are well-characterized. Six-microliter aliquots of PCR products were analyzed by gel electrophoresis with 1.5 % agarose in 1X TBE. Gels were stained with Midori Green stain and visualized in Gel imaging system FastGene FAS-DIGI PRO. A 100-bp DNA ladder was used as a marker. The PCR amplicons were purified using the kit NucleoSpin Gel and PCR clean-up. Subsequently, their concentration and DNA quality were determined by checking OD and running the samples on an agarose gel, respectively, and then were subjected to Sanger sequence analysis by CeMIA SA (http://cemia.eu/sangersequencing.html, accessed on 12 September 2022) **[99, 107**]. The set of primers used for sequencing were the same as those used in PCR. The sequences and chromatographs were interpreted using MEGA software (https://www.megasoftware.net/, accessed on 19 September 2022), and the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 18 September 2022) was used to identify antimicrobial resistance genes. DNA sequences were compared with reference antibiotic resistance genes from NCBI (https://www.ncbi.nlm.nih.gov/pathogens/refgene, accessed on 19 September 2022) and phylogenetic trees were constructed using the maximum likelihood method to investigate any possible correlations.

Table 2.2 ESBL genes PCR amplification program [103]			
Cycle step	**Temperature (°C)**	**Time**	**Number of cycles**
Initial denaturation	**94**	**5 min**	**1**
Denaturation	**94**	**25sec**	**30**
Annealing	**Melting Temparature (Tm)***	**40sec**	
Extension	**72**	**50 sec**	
Final elongation	**72**	**6 min**	**1**
***See Tm of specific genes in Table 2.6**			

Table 2.3 AmpC genes PCR amplification program [105]			
Cycle step	**Temperature (°C)**	**Time**	**Number of cycles**
Initial denaturation	**94**	**3 min**	**1**
Denaturation	**94**	**30 sec**	**25**
Annealing	**64**	**30 sec**	
Extension	**72**	**1 min**	
Final elongation	**72**	**7 min**	**1**

Table 2.4 Carbapenemase genes PCR amplification program [104]			
Cycle step	**Temperature (°C)**	**Time**	**Number of cycles**
Initial denaturation	**94**	**5 min**	**1**
Denaturation	**94**	**30 sec**	**30**
Annealing	**Melting Temparature (Tm)***	**30 sec**	
Extension	**72**	**1 min**	
Final elongation	**72**	**10 min**	**1**
***See Tm of specific genes in Table 2.6**			

Table 2.5 Dihydropteroate synthase gene (sul1) PCR amplification program [106]			
Cycle step	**Temperature (°C)**	**Time**	**Number of cycles**
Initial denaturation	**94**	**3 min**	**1**
Denaturation	**94**	**45 sec**	**30**
Annealing	**60**	**30 sec**	
Extension	**72**	**1.5 min**	
Final elongation	**72**	**2 min**	**1**

Table 2.6 Primer sets used for resistance genes detection						
		Target gene	Sequences 5'→ 3'	Melting temperature- Tm (oC)	Product size (bp)	Reference
β-lactamase genes	ESBL genes	BlaCTX-M-group 1-type	F_AAAAATCACTGCGCCAGTTC R_ AGCTTATTCATCGCCACGTT	55	415	[103]
		BlaCTX-M-group 2-type	F_ CGACGCTACCCCTGCTATT R_CCAGCGTCAGATTTTTCAGG		552	
		BlaCTX-M-group 9-type	F_CAAAGAGAGTGCAACGGATG R_ATTGGAAAGCGTTCATCACC		205	
		BlaSHV	F_AAGATCCACTATCGCCAGCAG R_ATTCAGTTCCGTTTCCCAGCGG	58	300	[102]
		BlaTEM	F_GAGTATTCAACATTTCCGTGTC R_TAATCAGTGAGGCACCTATCTC		850	
	AmpC type genes	BlaCMY-type	F_TGGCCAGAACTGACAGGCAAA R_TTTCTCCTGAACGTGGCTGGC	64	462	[105]
		BlaFOX-type	F_AACATGGGGTATCAGGGAGATG R_CAAAGCGCGTAACCGGATTGG		190	
		BlaDHA-type	F_AACTTTCACAGGTGTGCTGGGT R_CCGTACGCATACTGGCTTTGC		405	
		BlaACC-type	F_AACAGCCTCAGCAGCCGGTTA R_TTCGCCGCAATCATCCCTAGC		346	
		BlaMIR-type	F_TCGGTAAAGCCGATGTTGCGG R_CTTCCACTGCGGCTGCCAGTT		302	
	Carbapenemase genes	BlaOXA-48-type	F_TTGGTGGCATCGATTATCGG R_GAGCACTTCTTTTGTGATGGC	58	744	[102, 104]
		BlaVIM-type	F_AGTGGTGAGTATCCGACAG R_TCAATCTCCGCGAGAAG	52	212	[104]
		BlaNDM-type	F_TGGCAGCACACTTCCTATC R_AGATTGCCGAGCGACTTG	58	488	[104]
		BlaIMP-type	F_GGAATAGAGTGGCTTAACTC R_TCGGTTTAATAAAACAACCACC	56	232	[102]
		BlaKPC-type	F_CTGTCTTGTCTCTCATGGCC R_CCTCGCTGTRCTTGTCATCC	60	796	[104]
sulfonamide resistance gene		sul-1	F_GATTTTTCTTGAGCCCCGC R_TGGACCCAGATCCTTTACAGG	58	200	[106]

2.9 Molecular typing

2.9.1 Phylogrouping typing method

Materials/ reagents and equipment for triplex PCR

- Water for injection
- DreamTaq DNA Polymerase, 5 U/µL (Thermo Scientific™, EP0702)
- 10X DreamTaq Buffer (includes 20 mM MgCl2) (Thermo Scientific™, EP0702)
- Deoxynucleotide triphosphates (dNTPs) Bundle, 4x 100 mM (dATP, dCTP, dGTP, dTTP), (JENA BIOSCIENCE, NU-1005S)
- Primers of 100 µM stock solution (Invitrogen)
- *Escherichia coli* ATCC 25922 (Positive control)
- PCR Thermocycler 2720 (Applied Biosyststems, Thermo Fisher Scientific)
- Mini Centrifuge (MiniSpin ® Eppendorf, 22331)

Materials/ reagents and Equipement for PCR-product electrophoresis

- FastGene Agaroze (Nippon Genetics, Cat.:AG02)
- Midori Green Advance gels stain (Nippon Genetics Cat. No.: MG04)
- 10X Tris – Borate EDTA (TBE) stock solution: 900 mM Tris base, 900mM Boric acid and 20mM EDTA pH 8.0
- BlueJuice™ Gel Loading Buffer 10x (Invitrogen™ by Thermo Fisher Scientific, Cat. No: 10816015)
- DNA ladder (FastGene 100 bp DNA Marker, Nippon Genetics Cat. No. MWD100)
- Horizontal electrophoresis tank ClearSub L10 (Kisker Biotech)
- Gel imaging system FastGene FAS-DIGI PRO (Nippon Genetics)

Method

The Triplex PCR phylogrouping method utilizes the detection of chuA and yjaA genes and the DNA fragment TSPE4.C2 to classify *E. coli* isolates into four phylogenetic groups, A, B1, B2 and D, as per Clermont's schema [**108**].

The genomic DNA of *E. coli* bacterial isolates was amplified by triplex PCR using three pairs of primers simultaneously, targeted at three markers chuA, yjaA and TspE4.C2.

The DNA lysates the boiling method were performed as follow: a full loop of fresh pure cultures grown on Nutrient agar were resuspended in 250 µL water for injection, lysed by heating at 100 oC for 25 min and then were immediately put on ice for 10 min. The supernatant, which contains the whole bacterial genome, was harvested by centrifugation at 11.000 rpm for 10 min.

The PCR was performed with a standard protocol. Each reaction was carried out by using a 25 µl mixture containing 2.5µl of 10× DreamTaq buffer (includes 20mM MgCL2), 0.8 µM of each primer (initial concentration 20 µM), 0.2 mM of each deoxynucleoside triphosphate (initial concentration 10 mM), 1.5 U of DreamTaq polymerase, and 3µl of genomic DNA. The sequences and the other characteristics of primer pairs used are presented in **Table 2.7**. Negative controls (reaction lacking the template DNA) and a positive control (*Escherichia coli* ATCC 25922) were included in all performed amplifications. The PCR was performed under the following conditions: denaturation for 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and a final extension step of 7 min at 72°C [**108**]. Six-microliter aliquots of PCR products were analyzed by gel electrophoresis with 2% agarose in 1X TBE. Gels were stained with Midori Green stain and visualized in Gel imaging system FastGene FAS-DIGI PRO. A 100-bp DNA ladder was used as a marker.

This method was employed to investigate the correlation between the origin of the sample (clinical specimens, HWW, WWTP effluents, RWS1 and RWS2) and the phylogenetic groups, and to assess the possible association between groups and specific resistance profiles.

Table 2.7 Primer pairs used for Phylogrouping- triplex PCR [108]			
Target gene or locus	**Sequences 5'-→ 3'**	**Melting temperature- Tm (oC)**	**Product size (bp)**
chuA	**F_GACGAACCAACGGTCAGGAT** **R_TGCCGCCAGTACCAAAGACA**	**55**	**279**
YjaA	**F_TGAAGTGTCAGGAGACGCTG** **R_ATGGAGAATGCGTTCCTCAAC**		**211**
TspE4.C2	**F_GAGTAATGTCGGGGCATTCA** **R_CGCGCCAACAAAGTATTACG**		**152**

2.9.2. Pulsed field gel electrophoresis (PFGE)

Material and equipment

- Water for injection
- Ethylenediaminetetraacetic acid (EDTA) 0.5M pH 8.0
- EDTA 0.5M pH 9.0
- Tris-HCl 1M pH 7.4
- N-Lauroylsarcosine Sodium Salt (Sarcosyl) 10%
- Sodium Chloride (NaCl) Solution 5.0 M
- TE (Tris-EDTA): 10mM Tris-HCl, 1mM EDTA pH 8.0
- EC buffer: 6mM Tris-HCl, 1M NaCl, 100mM EDTA pH 9.0, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% sarkosyl
- ESP: 0.5M EDTA, 1% sarkosyl, pH 9.0
- Lysozyme 25mg/ml (Apollo Scientific, Cat. No BIL1028-10)
- Proteinase K 20 mg/ml (Invitrogen™, Cat No AM2544)
- Restriction endonuclease XbaI, 3000 U (Takara Bio, Cat. No 1093AH)
- Seakem Gold agarose (Lonza Bioscience, Cat. No 50150)
- TBE (Tris Borate EDTA) 5X: 0.9M Tris-HCl:0.9M Boric Acid: 0.02M EDTA pH 8.0
- Gel Red nucleic acid gels stain 10,000x in water (BIOTIUM, Cat. No 41003)
- Salmonella Braenderup H9812 (universal standard of DNA size in PFGE)
- Pulsed Field Gel Electrophoresis (PFGE) Systems (BIORAD)
- DOLPHIN – DOC imaging system (Wealtec Bioscience, Cat. No 1141004)

Method

The PFGE typing method is based on the comparison of profiles (PFGE patterns) generated following restriction digestion of bacterial DNA. This method was applied to 51 representative MDR isolates derived from different environments (6 clinical isolates, 17 from HWW, 13 from WWTP effluents, 8 from RSW1, and 7 from RSW2), which belong to different phylogenetic groups, in order to reveal their phylogenetic relationship based on the their PFGE patterns. PFGE was performed according to the pulse net protocol [**109**]. The isolates were cultured in nutrient agar overnight at 37 °C, and treated with lysozyme at 37 °C for 1 h and then with proteinase K at 56 °C for overnight incubation. After four washing steps, the DNA was digested using rare-cutting restriction endonuclease XbaI (30 units/reaction) at 37 °C overnight. The produced fragments of the digested genomic DNA were resolved on 1% agarose gels by electrophoretic current 'pulsed' in different directions for different lengths of time (PFGE system). The conditions for electrophoresis were as follows: 6 V/cm, 120°, Initial duration: 5 sec, Final duration: 55 sec, Total duration: 20h.

In this method the strain Salmonella Braenderup H9812 was utilized as both a DNA size marker and a quality control. Following electrophoresis, genomic profiles were visualized by staining with GelRed under UV light using a DOLPHIN – DOC imaging system. The final step involved evaluating the similarity of the strains through visual examination of the molecular fingerprints, based on the criteria established by Tenover et al [**110**].

2.9.3 Plasmid pattern- based typing

Materials and equipment

- EDTA 0.5 M EDTA pH 8.0
- Tris-HCl 1M pH 8.0
- TE Buffer: (50mM Tris-HCl, 10mM EDTA) pH 8.0
- Lysis Buffer: TE Buffer, 0.4% sodium dodecyl sulfate (SDS), pH 12.39-12.42
- Tris-HCl 2M pH 7.0
- NaCl 5M
- CH_3COONa 3M pH 5.2
- Phenol equilibrated, stabilized (PanReac AppliChem ITW Reagents, A1153)
- Water for injection
- 10X Tris – Borate EDTA (TBE) stock solution: 900 mM Tris base, 900mM Boric acid and 20mM EDTA pH 8.0 (TBE Buffer 0.5X)
- *E. coli* 39R861 (control strain)
- Refrigerated centrifuge (Mikro 22R, Hettich)

- FastGene Agaroze (Nippon Genetics, Cat.:AG02)
- Midori Green Advance gels stain (Nippon Genetics Cat. No.: MG04)
- BlueJuice™ Gel Loading Buffer 10x (Invitrogen™ by Thermo Fisher Scientific, Cat. No: 10816015)
- DNA ladder (FastGene 100 bp DNA Marker, Nippon Genetics Cat. No. MWD100)
- Horizontal electrophoresis tank ClearSub L10 (Kisker Biotech)
- Gel imaging system FastGene FAS-DIGI PRO (Nippon Genetics)

Method

Plasmid pattern- based typing was employed to the eighty-four (73 environmental and 11 clinical) isolates that were confirmed to be β-lactamase producers and were distributed in all phylogenetic groups. The application of this method aimed at: a) estimating the size of the plasmids they might harbor, and b) grouping the isolates according to their plasmid profiles and the resistance genes they carry. Furthermore, by comparing the generated plasmid profiles, it can be determined whether the bacterial isolates share common plasmids, indicating potential transmission or relatedness.

This method was carried out according to the protocol described by Portnoy et al [**111, 112**]. Initially, a full loop of fresh pure cultures grown on Nutrient agar was harvested and resuspended in 60 μl of TE buffer (pH 8). This suspension was then mixed with 600 μl of Lysis buffer [0.4% sodium dodecyl sulfate (SDS), pH 12.42]. The alkaline environment and SDS disrupted the cell membranes and denatured chromosomal DNA, while the plasmid DNA remained intact. Subsequently, 45 μl of 2 M Tris-HCl (pH 7) was added for neutralization, followed by 160 μl of 5M NaCl to precipitate the single- stranded chromosomal DNA. For further purification and to concentrate the plasmid DNA, phenol extraction was performed, followed by ethanol precipitation. After these steps, the sediment was dried, resuspended in 25 μl of TE buffer, and subjected to electrophoresis on a 0.72% agarose gel in 0.5X TBE buffer. The gels were stained with Midori Green and visualized using the FastGene FAS-DIGI PRO gel imaging system. The molecular weight of the purified plasmids (in Mega Daltons, MDa) was estimated by comparing them to known plasmids from the control strain *E. coli* 39R861, which contains plasmids of 98.0, 42.0, 23.9, 4.2 Mda and resulting plasmid profiles were compared across different isolates.

2.10 Statistical analysis

Pearson's chi-square test (or Fisher's exact test in case the expected values of any of the cells were below 5) was performed to examine the relationship between the phylogenetic groups and origin of the sample, and additionally between the phylogenetic groups and resistance profiles. The SPSS v.29 package was used for statistical analysis.

2.11 Transfer of resistance: conjugation assay

Materials

- LB Broth (Miller) (Neogen®, NCM0088A)
- 0.9% NaCl
- MacConkey Agar No.3 (Neogen® Culture Media, NCM0174B)
- Cefotaxime Sodium for injection 1 gram (SteriMax, ST-BQ212)
- Streptomycin injectable 25% (Neocell Pharmaceuticals)
- *Escherichia coli* 1R716 (recipient strain).

Method

The purpose of the conjugation assay was to investigate the potential transfer of resistance genes and to assess the frequency of plasmid transmission in selected isolates. Specifically, this method was applied to thirty three (27 environmental and 6 clinical) representative β-lactamase producing *E. coli* isolates from all geographical study sites with different plasmid profiles.

Conjugation experiments in broth as previously described [**113**] were performed to estimate the plasmid transfer frequency (also called conjugation rate) which parametrizes the horizontal spread of the studied plasmids carrying β-lactamases genes. *E. coli* 1R716 (lac-, ampicillin sensitive, streptomycin resistant) was used as recipient strain and the studied β-lactamase producing *E. coli* isolates (lac+, ampicillin resistant, streptomycin sensitive) as donors.

Briefly, both donor and recipient strains were inoculated in 10 mL of LB broth. After 24h incubation at 37 °C, donor and recipient suspensions were mixed in the same tube (co-culture) at a ratio of 1:1 and incubated at 37 °C for 18 h. Co-cultures were underwent on ten-fold serial dilutions (10-1 up to 10-6) and 100μl of each dilution were spread on selection MacConkey No3 agar plates containing a combination of streptomycin (STR, 1500 μg/mL) and cefotaxime (CTX, 0.4 μg/mL). This necessitated that all donor strains were pre-tested for susceptibility to streptomycin and recipient strain for susceptibility to cefotaxime. For the controls, 100μl of 10-6 diluted co-culture were inoculated on MacConkey No3 agar plates which contain no antibiotic (non-selection plates), in which both strains (donor and recipient) could grow on. Those non-selective plates were used to determine the conjugation efficiency by comparing growth to the selective plates.

The lac- colonies on selection plates were the potential transconjugants clones and the lac+ colonies on the non-selection plate provide the denominator for calculating the transfer frequency. Conjugation frequencies (CF) were calculated as follows:

$$CF = \frac{Number\ of\ lac-colonies\ (transconjugants)\ on\ selective\ plates\ x\ dilution\ factor}{Number\ of\ lac+colonies\ (donors)\ on\ non\ selective\ plates}$$

The lac- colonies that grew on the selective media were collected and tested for:

a) Their antibiotic suspectibility in β-lactams, aminoglycosides (including streptomycin), SXT and fluoroquinoles and

b) For the presence of β-lactamase genes via PCR on various transconjugant colonies per conjugation experiment.

Furthemore, transconjugants clones were submitted to plasmid analysis (according to the protocol described by Portnoy et al; **111, 112**], in order to determine the size of the transferred plasmids.

2.12 Plasmid sequencing, assembly, and annotation and bioinformatics analysis

Materials

- LB Broth (Miller) (Neogen®, NCM0088A)
- Cefotaxime Sodium for injection 1 gram (SteriMax, ST-BQ212)
- Streptomycin injectable 25% (Neocell Pharmaceuticals)
- Refrigerated Centrifuge (Kubota Model S700TR)
- Nucleobond Kit BAC 100/ 10 cart. (MACHEREY -NAGEL, Cat. No 740579)

Method

Initially, for the plasmid DNA extraction from the transconjugants, the following procedures were employed: a) high-density 500 ml liquid cultures in LB broth supplemented with streptomycin (1000 μg/ ml) and cefotaxime (0.4 μg/ ml), and b) the bacterial pellet obtained after centrifugation (at 3,200 rpm for 25 min) was processed using the NucleoBond BAC 100 kit for large construct plasmid DNA, following the manufacturer's instructions. This kit utilizes an optimized alkaline lysis procedure followed by anion-exchange chromatography to ensure high yields and purity of the plasmid DNA. The resulting DNA is suitable for downstream applications, including sequencing.

Sequencing of β-lactamase gene-carrying plasmids was conducted by Eurofins, using Oxford Nanopore Technology (ONT). Sequencing and the resulting reads are then subjected to quality filtering, assembly, and annotation using the Nanopore data analysis pipeline. The draft sequence of these plasmids was used for the characterisation of the β-lactamase genetic environment.

Acquired ARGs and other features in the plasmid DNA of each isolate were identified using the websever ResFinder 4.1 with a minimum coverage of 80% and a minimum identity of 95% as well as Proksee software. The PlasmidFinder bioinformatic tool was used for the identification of plasmid replicon types (incompatibility groups). Sequence similarity search was performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 15 July 2024) scans against the GenBank database. BLAST Ring Image Generator (BRIG) 0.9534 was used for plasmid comparisons and while Proksee for map construction.

Chapter 3

Experimental Part

Results

3.1 *E. coli* collection

The total number of *E. coli* colonies was determined by counting the number of characteristic colonies on the membrane filter according to ISO 9308.01-1:2017. A total of 610 colonies presumptive of *E. coli* (identified by their blue-violet color in CCA) were initially collected. Out of the 610 colonies, 502 (171 from WWTP, 105 from semi-treated HWW, 163 from RWS1 and 63 from RWS2 samples) were finally confirmed as being *E. coli* using the gold standard procedures [**ISO 9308.01-1:2017**] and molecular uidA confirmatory test [**91**]. In more detail, of the 502 confirmed E. coli isolates, 296 (92 from WWTP, 73 from HWW, 91 from RWS1 and 40 from RWS2 samples) were collected from CCA culture media without AMP and 206 (79 from WWTP, 32 from HWW, 72 from RWS1 and 23 from RWS2 samples) were collected from CCA/AMP. Regarding the clinical collection, a total of 139 *E. coli* isolates were identified and confirmed, with 104 derived from urine, 30 from blood and 5 from patients' tissue.

3.2. Antimicrobial susceptibility profiles and assessment of resistance mechanisms

Considering that *E. coli* has no intrinsic resistance mechanisms, all of the isolates (environmental and clinical) were classified into specific sub-categories. Regarding the environmental isolates, 40.4% (203/502) were characterized as WT, 2.8% (14/502) were characterized as N-WT, 36.5% (183/502) were characterized as R and 20.3% (102/502) were characterized as MDR. Regarding the clinical isolates, 40% (56/139) were characterized as S, 46% (64/139) were characterized as R and 14% (19/139) were characterized as MDR. The results of the antimicrobial susceptibility tests (antibiograms) and the characteristics of all isolates are presented in **Table 3.1** while the data for the characterization of the resistance profiles of the environmental and clinical samples are summarized in **Table 3.2**.

The resistance frequencies of the 502 environmental and 139 clinical isolates in all of the tested antibiotics are presented in **Figure 3.1**. Resistance to penicillins (AMP and PIP) was the most frequent among all of the environmental and clinical isolates, followed by resistance to AMC.

In more detail, 55% (275/502) of the environmental isolates exhibited resistance to AMP, 53% (267/502) exhibited resistance to PIP and 33% (164/502) exhibited resistance to AMC. A high resistance rate to quinolones (24.9%; 125/502) was also observed and the majority of the resistant isolates were derived from HWW (33.6%; 42/125) (**Figure 3.1**).

Regarding the 139 clinical isolates, 40% (55/139) presented resistance to AMP, 33% (46/139) presented resistance to PIP and 25% (35/139) presented resistance to AMC. The number of different antibiotic categories in which environmental and clinical MDR isolates presented resistance is shown in **Table 3.3**.

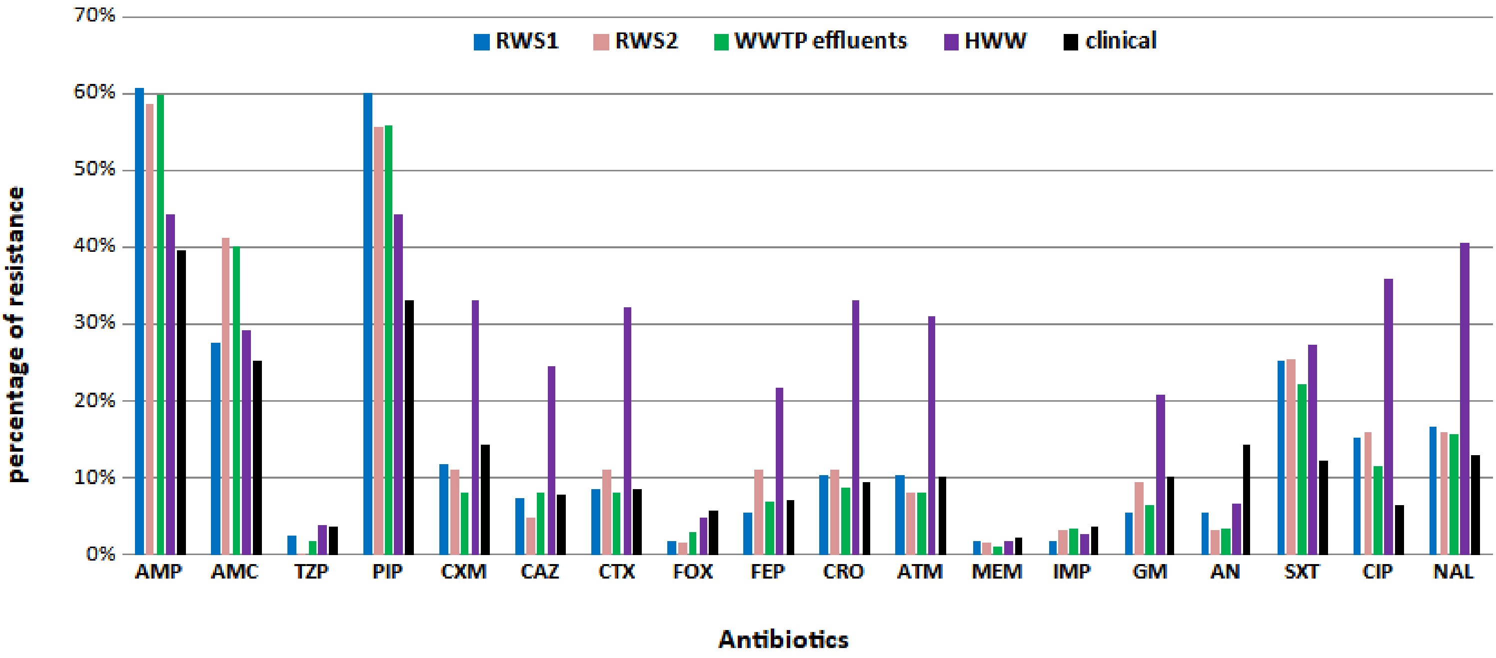


Figure 3.1 The frequency rate of resistance to each antibiotic per origin of sample.

[Abbreviations: AMP, ampicillin; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/ tazobactam; PIP, piperacillin; CXM, cefuroxime; CAZ, ceftazidime; CTX, cefotaxime; FOX, cefoxitin; FEP, cefepime; CRO, ceftriaxone; ATM, aztreonam; MEM, meropenem; IMP, imipenem; GM, gentamicin; AN, amikacin; SXT, sulfamethoxazole-trimethoprim; CIP, ciprofloxacin; NAL, nalidixic acid. HWW, hospital wastewater; WWTP, wastewater treatment plant; RWS1, river water site 1; RWS2, river water site 2]

Table 3.1 All environmental and clinical *E.coli* isolates and their characteristics (sampling season, type of sample, sampling site, resistance pattern and profile and phylogenetic group)

Isolates	Sampling Season	Type of Sample	Sampling Site	Phylogenetic group	Resistance Pattern	Resistance Profile	DDST
293	Summer 2019	Treated Wastewater	WWTP outlet	A	AMP, AMC	R	
294	Summer 2019	Treated Wastewater	WWTP outlet	A		WT	
295	Summer 2019	Treated Wastewater	WWTP outlet	A		WT	
296	Summer 2019	Treated Wastewater	WWTP outlet	A	AMP, AMC, CIP, NAL	R	
297	Summer 2019	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, TCC, CAZ, CTX, CRO, ATM, AN	MDR	+
298	Summer 2019	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, TCC	R	
299	Summer 2019	Treated Wastewater	WWTP outlet	B1	AMP, AMC, PIP, TCC, NAL	R	
300	Summer 2019	Treated Wastewater	WWTP outlet	A	AMP, AMC, TZP, PIP, TCC	R	
301	Summer 2019	Treated Wastewater	WWTP outlet	B2	AMP, AMC	R	
302	Summer 2019	Treated Wastewater	WWTP outlet	B1	AMP, AMC, PIP, TCC	R	
303	Summer 2019	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, TCC, SXT	R	
304	Summer 2019	Treated Wastewater	WWTP outlet	A		WT	
305	Summer 2019	River water	RWS2	A		WT	
306	Summer 2019	River water	RWS2	B1	AMP, AMC, PIP, TCC	R	
307	Summer 2019	River water	RWS2	A		WT	
308	Summer 2019	River water	RWS2	A		WT	
309	Summer 2019	River water	RWS2	B1	AMP, AMC	R	
310	Summer 2019	River water	RWS2	A	AMP, AMC	R	
311	Summer 2019	River water	RWS1	D	AMP, AMC, PIP, TCC	R	
312	Summer 2019	River water	RWS1	A		WT	
313	Summer 2019	River water	RWS1	A	AMP, AMC, PIP, TCC	R	
314	Summer 2019	River water	RWS1	A		WT	
315	Summer 2019	River water	RWS1	A		WT	
316	Summer 2019	River water	RWS2	B2	SXT	N-WT	
317	Autumn 2019	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, NAL	R	
318	Autumn 2019	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, CIP, NAL	R	
319	Autumn 2019	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, SXT	R	
320	Autumn 2019	Treated Wastewater	WWTP outlet	A	AMP, PIP	R	
321	Autumn 2019	Treated Wastewater	WWTP outlet	A	AMP, PIP	R	
322	Autumn 2019	Treated Wastewater	WWTP outlet	B1	AMP, PIP	R	
323	Autumn 2019	Treated Wastewater	WWTP outlet	B1	AMP, AMC, PIP	R	
324	Autumn 2019	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, TCC	R	
325	Autumn 2019	Treated Wastewater	WWTP outlet	B2	AMP, PIP	R	
326	Autumn 2019	Treated Wastewater	WWTP outlet	B2	AMP, PIP, MEM, IMP	R	-
327	Autumn 2019	Treated Wastewater	WWTP outlet	B2		WT	
328	Autumn 2019	Treated Wastewater	WWTP outlet	B2	AMP, AMC, FOX, AN, NAL	MDR	
329	Autumn 2019	Treated Wastewater	WWTP outlet	D	AMP, AMC, PIP, TCC	R	
330	Autumn 2019	Treated Wastewater	WWTP outlet	A		WT	
331	Autumn 2019	Treated Wastewater	WWTP outlet	B2		WT	
332	Autumn 2019	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP , GM, AN, SXT, CIP, NAL	MDR	
333	Autumn 2019	Treated Wastewater	WWTP outlet	A		WT	
334	Autumn 2019	Treated Wastewater	WWTP outlet	B1		WT	
335	Autumn 2019	Treated Wastewater	WWTP outlet	A		WT	
337	Autumn 2019	Treated Wastewater	WWTP outlet	A	CIP, NAL	R	
338	Autumn 2019	Treated Wastewater	WWTP outlet	A		WT	
339	Autumn 2019	Treated Wastewater	WWTP outlet	D		WT	
340	Autumn 2019	Treated Wastewater	WWTP outlet	B2	AMP, AMC, TCC, FOX, NAL	MDR	
341	Autumn 2019	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, GM	R	
344	Autumn 2019	HWW	Septic tank	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, CIP, NAL	MDR	+
345	Autumn 2019	HWW	Septic tank	A	AMP, PIP, CAZ, CTX, CRO, ATM, NAL	MDR	+
347	Autumn 2019	HWW	Septic tank	A	AMP, AMC, SXT	R	
348	Autumn 2019	HWW	Septic tank	A		WT	
349	Autumn 2019	HWW	Septic tank	A	SXT	N-WT	
350	Autumn 2019	HWW	Septic tank	A		WT	
352	Autumn 2019	HWW	Septic tank	B2	AMP, AMC, PIP, TCC, AN, SXT, NAL	MDR	
355	Autumn 2019	River water	RWS1	A	AMP, AMC, PIP, TCC, NAL	R	
356	Autumn 2019	River water	RWS1	D	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM	MDR	+
357	Autumn 2019	River water	RWS1	B2	AMP, AMC, PIP	R	
358	Autumn 2019	River water	RWS1	A	AMP, AMC, PIP, NAL	R	
359	Autumn 2019	River water	RWS1	A	AMP, AMC, PIP, NAL	R	
361	Autumn 2019	River water	RWS1	B1		WT	
362	Autumn 2019	River water	RWS1	A		WT	
363	Autumn 2019	River water	RWS1	A	AMP, AMC, PIP	R	
364	Autumn 2019	River water	RWS1	B1		WT	
365	Autumn 2019	River water	RWS1	A		WT	
367	Autumn 2019	River water	RWS1	A	AMP, AMC, PIP, CXM, CAZ, FOX, CRO, ATM	MDR	-
368	Autumn 2019	River water	RWS1	A		WT	
369	Autumn 2019	River water	RWS2	A	AMP, PIP , NAL	R	
370	Autumn 2019	River water	RWS2	A	AMP, AMC, PIP, NAL	R	
371	Autumn 2019	River water	RWS2	A	AMP, AMC, PIP, SXT, NAL	R	
372	Autumn 2019	River water	RWS2	D	AMP, AMC, PIP	R	
373	Autumn 2019	River water	RWS2	A	AMP, AMC, PIP, SXT	R	
374	Autumn 2019	River water	RWS2	A	AMP, AMC, PIP	R	
375	Autumn 2019	River water	RWS2	A	AMP, AMC, PIP, SXT, CIP, NAL	MDR	
376	Autumn 2019	River water	RWS2	A	AMP, AMC, PIP, SXT	R	
377	Autumn 2019	River water	RWS2	B2	AMP, PIP	R	
378	Autumn 2019	River water	RWS2	B2	AMP, AMC, PIP, CIP, NAL	R	
379	Autumn 2019	River water	RWS2	B1		WT	
382	Autumn 2019	River water	RWS2	B1		WT	

383	Winter 2020	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, TCC	R	
384	Winter 2020	Treated Wastewater	WWTP outlet	B2	AMP, AMC, PIP, TCC	R	
385	Winter 2020	Treated Wastewater	WWTP outlet	B2	AMP, AMC, PIP, TCC	R	
386	Winter 2020	Treated Wastewater	WWTP outlet	D	AMP, AMC, PIP, TCC, AN, SXT	MDR	
387	Winter 2020	Treated Wastewater	WWTP outlet	B2	AMP, AMC, PIP, TCC	R	
388	Winter 2020	Treated Wastewater	WWTP outlet	B2	AMP, AMC, PIP, TCC	R	
389	Winter 2020	Treated Wastewater	WWTP outlet	B2	AMP, AMC, PIP, TCC	R	
391	Winter 2020	Treated Wastewater	WWTP outlet	A		WT	
392	Winter 2020	Treated Wastewater	WWTP outlet	A		WT	
393	Winter 2020	Treated Wastewater	WWTP outlet	A		WT	
394	Winter 2020	Treated Wastewater	WWTP outlet	A	AMP	N-WT	
396	Winter 2020	Treated Wastewater	WWTP outlet	B2	AMP, AMC, PIP, TCC	R	
397	Winter 2020	River water	RWS2	B2		WT	
398	Winter 2020	River water	RWS2	A		WT	
399	Winter 2020	River water	RWS2	B2		WT	
400	Winter 2020	River water	RWS2	A		WT	
401	Winter 2020	River water	RWS2	A		WT	
402	Winter 2020	River water	RWS1	B2	AMP, PIP, NAL	R	
403	Winter 2020	River water	RWS1	B2	AMP, AMC, PIP, AN	R	
404	Winter 2020	River water	RWS1	A	AMP, AMC, PIP, TZP, TCC, SXT, CIP, NAL	MDR	
404 a	Winter 2020	River water	RWS1	A	AMP, AMC, PIP, AN, SXT, CIP, NAL	MDR	
405	Winter 2020	River water	RWS1	D	AMP, AMC, PIP, CXM, CTX, CRO, ATM, CIP, NAL	MDR	+
406	Winter 2020	River water	RWS1	D	AMP, AMC, PIP, SXT, NAL	R	
407	Winter 2020	River water	RWS1	A	AMP, PIP, SXT	R	
408	Winter 2020	River water	RWS1	D	AMP, AMC, TZP, PIP, TCC, CXM, CAZ, CTX, FEP, CRO, ATM, MEM, IMP, SXT	MDR	+
409	Winter 2020	River water	RWS2	A	AMP, AMC, PIP, SXT, CIP, NAL	MDR	
410	Winter 2020	River water	RWS1	D	AMP, PIP	R	
412	Winter 2020	River water	RWS1	A	AMP, AMC, PIP	R	
413	Winter 2020	River water	RWS1	A	AMP, AMC, PIP	R	
414	Winter 2020	River water	RWS1	A	AMP, AMC, PIP	R	
415	Winter 2020	River water	RWS1	B2	AMP, PIP, SXT	R	
416	Winter 2020	River water	RWS2	B1	AMP, PIP, SXT	R	
417	Winter 2020	River water	RWS2	B1		WT	
418	Winter 2020	River water	RWS2	B1		WT	
419	Winter 2020	River water	RWS1	A	AMP, AMC, PIP, SXT	R	
420	Winter 2020	River water	RWS2	B1		WT	
421	Winter 2020	River water	RWS2	D		WT	
422	Winter 2020	River water	RWS2	B1		WT	
426	Winter 2020	HWW	Septic tank	D	AMP, AMC, PIP, CXM, CAZ, CTX, CRO, ATM, CIP, NAL	MDR	+
427	Winter 2020	HWW	Septic tank	B2	AMP, AMC, PIP, CXM, CAZ, CTX, CRO, ATM, AN, CIP, NAL	MDR	+
431	Winter 2020	HWW	Septic tank	B2	AMP, PIP, CXM, CAZ, CTX, CRO, ATM, AN, CIP, NAL	MDR	+
434	Winter 2020	HWW	Septic tank	B2	AMP, AMC, PIP, CAZ, CTX, CRO, ATM, CIP, NAL	MDR	+
436	Winter 2020	HWW	Septic tank	B2	AMP, AMC, PIP, CXM, CAZ, CTX, CRO, ATM, AN, CIP, NAL	MDR	+
439	Winter 2020	HWW	Septic tank	B2		WT	
442	Winter 2020	HWW	Septic tank	A	AMP, AMC, NAL	R	
445	Winter 2020	HWW	Septic tank	B1		WT	
446	Winter 2020	HWW	Septic tank	B1		WT	
447	Winter 2020	HWW	Septic tank	B2	AMP, AMC, CXM, NAL	R	
448	Winter 2020	HWW	Septic tank	B1		WT	
450	Summer 2020	River water	RWS1	A		WT	
451	Summer 2020	River water	RWS1	A		WT	
452	Summer 2020	River water	RWS1	A		WT	
453	Summer 2020	River water	RWS1	A	AMP, AMC, PIP	R	
454	Summer 2020	River water	RWS1	B2		WT	
455	Summer 2020	River water	RWS1	B2	AMP, AMC, PIP	R	
456	Summer 2020	River water	RWS1	A	AMP, PIP.	R	
457	Summer 2020	River water	RWS1	A		WT	
458	Summer 2020	River water	RWS1	A	AMP, PIP, SXT, CIP, NAL	R	
459	Summer 2020	River water	RWS1	A		WT	
460	Summer 2020	River water	RWS1	B2		WT	
461	Summer 2020	River water	RWS1	A		WT	
462	Summer 2020	River water	RWS1	A		WT	
463	Summer 2020	River water	RWS1	B2		WT	
464	Summer 2020	River water	RWS1	B1		WT	
465	Summer 2020	River water	RWS1	A		WT	
466	Summer 2020	River water	RWS1	A	AMP, AMC, PIP, SXT, CIP, NAL	MDR	
467	Summer 2020	River water	RWS1	D	AMP, PIP, SXT, CIP, NAL	R	
468	Summer 2020	River water	RWS1	B2	AMP, PIP, AN	R	
469	Summer 2020	River water	RWS1	B2	AMP, AMC, PIP	R	
470	Summer 2020	River water	RWS1	A	AMP, PIP, CIP, NAL	R	
471	Summer 2020	River water	RWS1	B1	AMP, AMC, PIP, GM, SXT	MDR	
472	Summer 2020	River water	RWS1	A	AMP, AMC, PIP, CXM, CTX, CRO, ATM, NAL	MDR	+
473	Summer 2020	River water	RWS1	A	AMP, AMC, PIP	R	
474	Summer 2020	River water	RWS1	A	AMP, AMC, PIP	R	
475	Summer 2020	River water	RWS1	A	AMP, PIP, CIP, NAL	R	
476	Summer 2020	River water	RWS1	A	AMP, PIP, SXT, CIP, NAL	R	
477	Summer 2020	River water	RWS1	B1	AMP, PIP, CXM, CAZ, CRO, ATM, CIP, NAL	MDR	+
478	Summer 2020	River water	RWS1	A	AMP, AMC, PIP, AN, SXT	MDR	
479	Summer 2020	River water	RWS1	A	AMP, AMC, PIP	R	
481	Summer 2020	River water	RWS1	B1	AMP, AMC, PIP	R	
482	Summer 2020	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP	R	
483	Summer 2020	Treated Wastewater	WWTP outlet	B2	AMP, AMC, PIP, AN, CIP, NAL	MDR	
484	Summer 2020	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, GN, SXT	MDR	

485	Summer 2020	Treated Wastewater	WWTP outlet	D	AMP, AMC, PIP,	R	
486	Summer 2020	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP	R	
487	Summer 2020	Treated Wastewater	WWTP outlet	D	AMP, AMC, PIP, NAL	R	
488	Summer 2020	Treated Wastewater	WWTP outlet	B2	AMP, AMC, PIP, CIP, NAL	R	
489	Summer 2020	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP	R	
490	Summer 2020	Treated Wastewater	WWTP outlet	D	AMP, AMC, PIP	R	
491	Summer 2020	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, CXM, CTX, CRO, FEP, ATM, NAL	MDR	+
492	Summer 2020	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, SXT	R	
493	Summer 2020	Treated Wastewater	WWTP outlet	B2	AMP, AMC, PIP, SXT	R	
494	Summer 2020	Treated Wastewater	WWTP outlet	B1	AMP, AMC, PIP, CXM, CTX, CRO, SXT	MDR	+
495	Summer 2020	Treated Wastewater	WWTP outlet	A	AMP, PIP	R	
497	Summer 2020	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, NAL	MDR	+
500	Summer 2020	Treated Wastewater	WWTP outlet	D	AMP, AMC, PIP, SXT, CIP, NAL	MDR	
501	Summer 2020	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, GM, SXT	MDR	
505	Summer 2020	Treated Wastewater	WWTP outlet	A	AMP, PIP	R	
506	Summer 2020	Treated Wastewater	WWTP outlet	D	AMP, PIP, CXM, CAZ, CTX, FEP, ATM, SXT, NAL	MDR	+
508	Summer 2020	Treated Wastewater	WWTP outlet	D	AMP, PIP, CIP, NAL	R	
510	Summer 2020	Treated Wastewater	WWTP outlet	B2	AMP, PIP, CXM, CTX, CRO, SXT, CIP, NAL	MDR	+
511	Summer 2020	Treated Wastewater	WWTP outlet	B2		WT	
512	Summer 2020	Treated Wastewater	WWTP outlet	A		WT	
513	Summer 2020	Treated Wastewater	WWTP outlet	B2		WT	
514	Summer 2020	Treated Wastewater	WWTP outlet	D		WT	
516	Summer 2020	Treated Wastewater	WWTP outlet	A		WT	
517	Summer 2020	Treated Wastewater	WWTP outlet	B1		WT	
518	Summer 2020	Treated Wastewater	WWTP outlet	A	CAZ	N-WT	
519	Summer 2020	Treated Wastewater	WWTP outlet	D	AMP, PIP, SXT, CIP, NAL	R	
520	Summer 2020	Treated Wastewater	WWTP outlet	A	CIP, NAL	R	
522	Summer 2020	Treated Wastewater	WWTP outlet	A		WT	
523	Summer 2020	Treated Wastewater	WWTP outlet	B1		WT	
525	Summer 2020	Treated Wastewater	WWTP outlet	A		WT	
526	Summer 2020	Treated Wastewater	WWTP outlet	A		WT	
527	Summer 2020	Treated Wastewater	WWTP outlet	B1	NAL	N-WT	
528	Summer 2020	Treated Wastewater	WWTP outlet	A		WT	
529	Summer 2020	Treated Wastewater	WWTP outlet	A		WT	
530	Summer 2020	Treated Wastewater	WWTP outlet	B1		WT	
531	Summer 2020	Treated Wastewater	WWTP outlet	B1		WT	
532	Summer 2020	Treated Wastewater	WWTP outlet	A		WT	
533	Summer 2020	Treated Wastewater	WWTP outlet	B2		WT	
534	Summer 2020	Treated Wastewater	WWTP outlet	D		WT	
535	Summer 2020	Treated Wastewater	WWTP outlet	B2		WT	
536	Summer 2020	Treated Wastewater	WWTP outlet	B2		WT	
537	Summer 2020	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP	R	
538	Summer 2020	Treated Wastewater	WWTP outlet	B1		WT	
539	Summer 2020	Treated Wastewater	WWTP outlet	A		WT	
540	Summer 2020	HWW	Septic tank	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	+
542	Summer 2020	HWW	Septic tank	A	AMP, AMC, PIP, CXM, CTX, CRO, CIP, NAL	MDR	+
543	Summer 2020	HWW	Septic tank	A	AMP, AMC, PIP, CXM, CTX, CRO, FEP, ATM, GN, SXT, CIP, NAL	MDR	+
545	Summer 2020	HWW	Septic tank	D	AMP, AMC, PIP, SXT	R	
546	Summer 2020	HWW	Septic tank	B2	AMP, AMC, PIP, CXM, CAZ, CTX, CRO, ATM, NAL	MDR	+
550	Summer 2020	HWW	Septic tank	A	AMP, AMC, TZP, PIP, CXM, FOX, FEP, GM, SXT, CIP, NAL	MDR	-
552	Summer 2020	HWW	Septic tank	A	AMP, AMC, PIP, CXM,CAZ, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	+
553	Summer 2020	HWW	Septic tank	A	AMP, AMC, PIP, CXM, CTX, CRO, ATM, SXT, CIP, NAL	MDR	+
555	Summer 2020	HWW	Septic tank	A	AMP, AMC, PIP, CXM,CAZ, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	+
556	Summer 2020	HWW	Septic tank	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, CIP, NAL	MDR	+
557	Summer 2020	HWW	Septic tank	A	AMP, AMC, PIP, CXM, CTX, FEP, CRO, ATM, GN, SXT, CIP, NAL	MDR	+
558	Summer 2020	HWW	Septic tank	A	AMP, AMC, PIP, CXM, CTX, FEP, CRO, ATM, GN, SXT, CIP, NAL	MDR	+
560	Summer 2020	HWW	Septic tank	A		WT	
561	Summer 2020	HWW	Septic tank	A		WT	
562	Summer 2020	HWW	Septic tank	A		WT	
563	Summer 2020	HWW	Septic tank	A		WT	
566	Summer 2020	HWW	Septic tank	B1		WT	
567	Summer 2020	HWW	Septic tank	A		WT	
568	Summer 2020	HWW	Septic tank	A		WT	
569	Summer 2020	HWW	Septic tank	A	FOX	N-WT	
570	Summer 2020	HWW	Septic tank	D		WT	
571	Summer 2020	HWW	Septic tank	B2		WT	
572	Summer 2020	HWW	Septic tank	A		WT	
573	Summer 2020	HWW	Septic tank	A	NAL	N-WT	
574	Summer 2020	HWW	Septic tank	B2		WT	
575	Summer 2020	HWW	Septic tank	B1		WT	
576	Summer 2020	HWW	Septic tank	B2	AMP, AMC, PIP, SXT	R	
577	Summer 2020	HWW	Septic tank	B1		WT	
578	Summer 2020	HWW	Septic tank	A		WT	
579	Summer 2020	HWW	Septic tank	D		WT	
580	Summer 2020	HWW	Septic tank	D		WT	
581	Summer 2020	HWW	Septic tank	A	AMP, AMC, TZP, PIP, CXM, FOX, FEP, GM, SXT, CIP, NAL	MDR	-
588	Autumn 2020	River water	RWS1	D	AMP, PIP	R	
589	Autumn 2020	River water	RWS1	B2	AMP, PIP	R	
590	Autumn 2020	River water	RWS1	B2	AMP, PIP, SXT	R	
591	Autumn 2020	River water	RWS1	B2	AMP, PIP	R	
592	Autumn 2020	River water	RWS1	A	AMP, PIP, SXT	R	
594	Autumn 2020	River water	RWS1	B1	AMP, PIP, CXM, CAZ, CTX, CRO, ATM, CIP, NAL	MDR	+
595	Autumn 2020	River water	RWS1	B2	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM	MDR	+

596	Autumn 2020	River water	RWS1	B2	AMP, AMC, PIP, SXT	R	
597	Autumn 2020	River water	RWS1	B1	AMP, PIP, SXT	R	
598	Autumn 2020	River water	RWS1	D	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	+
599	Autumn 2020	River water	RWS1	A	AMP, PIP, AN, CIP, NAL	R	
600	Autumn 2020	River water	RWS1	B2	AMP, PIP	R	
601	Autumn 2020	River water	RWS1	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO	R	+
602	Autumn 2020	River water	RWS1	A	AMP, PIP, CXM, CAZ, CRO, ATM	MDR	+
603	Autumn 2020	River water	RWS1	B2	AMP, AMC, PIP, GM, AN, SXT	MDR	
604	Autumn 2020	River water	RWS1	A	AMP, PIP, SXT	R	
605	Autumn 2020	River water	RWS1	A	AMP, PIP	R	
606	Autumn 2020	River water	RWS1	A	AMP, AMC, PIP, SXT, CIP, NAL	MDR	
607	Autumn 2020	River water	RWS1	B1	AMP, AMC, PIP, GM, AN, SXT, CIP, NAL	MDR	
608	Autumn 2020	River water	RWS1	B2	AMP, PIP, SXT	R	
609	Autumn 2020	River water	RWS1	D	AMP, PIP	R	
610	Autumn 2020	River water	RWS1	D	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, NAL	MDR	+
611	Autumn 2020	River water	RWS1	A	AMP, PIP, CXM, CTX, FEP, CRO	R	+
612	Autumn 2020	River water	RWS2	D	AMP, AMC, PIP, NAL	R	
613	Autumn 2020	River water	RWS2	D	AMP, AMC, PIP	R	
614	Autumn 2020	River water	RWS2	A	AMP, PIP, CXM, CTX, FEP, CRO	R	+
615	Autumn 2020	River water	RWS2	A	AMP, PIP, GM	R	
616	Autumn 2020	River water	RWS2	A	AMP, PIP, CXM, CTX, FEP, CRO	R	+
617	Autumn 2020	River water	RWS2	A	AMP, AMC, PIP	R	
618	Autumn 2020	River water	RWS2	A	AMP, AMC, PIP, CXM, CAZ ,CTX, FEP, CRO, ATM	MDR	+
619	Autumn 2020	River water	RWS2	A	AMP, AMC, PIP	R	
620	Autumn 2020	River water	RWS2	A	AMP, PIP, CXM, CTX, FEP, CRO, ATM, AN	MDR	+
623	Autumn 2020	River water	RWS2	A	SXT, CIP, NAL	R	
624	Autumn 2020	River water	RWS2	B2		WT	
626	Autumn 2020	River water	RWS2	A		WT	
628	Autumn 2020	River water	RWS2	A	GM	N-WT	
629	Autumn 2020	River water	RWS2	A		WT	
630	Autumn 2020	River water	RWS2	A	AMP, AMC, PIP, CXM, CTX, FEP, CRO, ATM, AN	MDR	+
632	Autumn 2020	River water	RWS1	A	AMP, PIP, CIP, NAL	R	
633	Autumn 2020	Treated Wastewater	WWTP outlet	A	AMP, PIP, GM	R	
634	Autumn 2020	Treated Wastewater	WWTP outlet	B2	AMP, AMC, PIP, NAL	R	
635	Autumn 2020	Treated Wastewater	WWTP outlet	D	AMP, AMC, PIP	R	
636	Autumn 2020	Treated Wastewater	WWTP outlet	D	AMP, AMC, PIP	R	
637	Autumn 2020	Treated Wastewater	WWTP outlet	B1	AMP, AMC, PIP	R	
638	Autumn 2020	Treated Wastewater	WWTP outlet	D	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, SXT, NAL	MDR	+
639	Autumn 2020	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, SXT	R	
640	Autumn 2020	Treated Wastewater	WWTP outlet	A	AMP, PIP, SXT, CIP, NAL	R	
641	Autumn 2020	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, SXT, CIP, NAL	MDR	
642	Autumn 2020	Treated Wastewater	WWTP outlet	B1	AMP, AMC, TZP, PIP, SXT	R	
643	Autumn 2020	Treated Wastewater	WWTP outlet	B1	AMP, AMC, PIP, GM	R	
644	Autumn 2020	Treated Wastewater	WWTP outlet	D	AMP, AMC, PIP	R	
645	Autumn 2020	Treated Wastewater	WWTP outlet	D	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, NAL	MDR	+
646	Autumn 2020	Treated Wastewater	WWTP outlet	B2	AMP, AMC, PIP	R	
647	Autumn 2020	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP	R	
648	Autumn 2020	Treated Wastewater	WWTP outlet	B2	AMP, AMC, PIP	R	
649	Autumn 2020	Treated Wastewater	WWTP outlet	D	AMP, AMC, PIP, CIP, NAL	R	
650	Autumn 2020	Treated Wastewater	WWTP outlet	B1	AMP, AMC, PIP, GM	R	
651	Autumn 2020	Treated Wastewater	WWTP outlet	D	AMP, AMC, PIP	R	
652	Autumn 2020	Treated Wastewater	WWTP outlet	D	AMP, PIP, GM, SXT, CIP, NAL	MDR	
653	Autumn 2020	Treated Wastewater	WWTP outlet	B1		WT	
654	Autumn 2020	Treated Wastewater	WWTP outlet	A		WT	
655	Autumn 2020	Treated Wastewater	WWTP outlet	D	AMP, PIP	R	
656	Autumn 2020	Treated Wastewater	WWTP outlet	D	SXT	N-WT	
658	Autumn 2020	Treated Wastewater	WWTP outlet	B1		WT	
659	Autumn 2020	Treated Wastewater	WWTP outlet	D	AMP, PIP, GM, SXT, CIP, NAL	MDR	
660	Autumn 2020	Treated Wastewater	WWTP outlet	A		WT	
661	Autumn 2020	Treated Wastewater	WWTP outlet	A		WT	
662	Autumn 2020	Treated Wastewater	WWTP outlet	B2		WT	
663	Autumn 2020	Treated Wastewater	WWTP outlet	D		WT	
664	Autumn 2020	Treated Wastewater	WWTP outlet	B2		WT	
665	Autumn 2020	Treated Wastewater	WWTP outlet	A		WT	
666	Autumn 2020	Treated Wastewater	WWTP outlet	A		WT	
667	Autumn 2020	Treated Wastewater	WWTP outlet	B1		WT	
668	Autumn 2020	Treated Wastewater	WWTP outlet	B1		WT	
669	Autumn 2020	Treated Wastewater	WWTP outlet	B1		WT	
670	Autumn 2020	HWW	Septic tank	A		WT	
671	Autumn 2020	HWW	Septic tank	A	CIP, NAL	R	
672	Autumn 2020	HWW	Septic tank	B2		WT	
673	Autumn 2020	HWW	Septic tank	A	AMP, PIP, CAZ, CRO, ATM, NAL	MDR	+
674	Autumn 2020	HWW	Septic tank	A	CIP, NAL	R	
675	Autumn 2020	HWW	Septic tank	A		WT	
676	Autumn 2020	HWW	Septic tank	A		WT	
677	Autumn 2020	HWW	Septic tank	A		WT	
678	Autumn 2020	HWW	Septic tank	A	AMP, AMC, PIP, CXM, CAZ ,CTX, FEP, CRO, ATM, GN, SXT, CIP, NAL	MDR	+
679	Autumn 2020	HWW	Septic tank	A	CIP, NAL	R	
680	Autumn 2020	HWW	Septic tank	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GN, SXT, CIP, NAL	MDR	+
681	Autumn 2020	HWW	Septic tank	A	AMP, PIP, CXM, CTX, FEP, CRO, ATM, GN, SXT, CIP, NAL	MDR	+
682	Autumn 2020	HWW	Septic tank	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GN, SXT, CIP, NAL	MDR	+
683	Autumn 2020	HWW	Septic tank	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GN, SXT, CIP, NAL	MDR	+
684	Autumn 2020	HWW	Septic tank	A	AMP, PIP, CXM, CTX, CRO, ATM, GN, SXT, CIP, NAL	MDR	+

685	Autumn 2020	HWW	Septic tank	A	AMP, PIP, CXM, CTX, CRO, ATM, GN, SXT, CIP, NAL	MDR	+
686	Autumn 2020	HWW	Septic tank	A	AMP, AMC, PIP, CXM, CTX, FEP, CRO, ATM, GN, SXT, CIP, NAL	MDR	+
690	Autumn 2020	HWW	Septic tank	A	NAL	N-WT	
691	Autumn 2020	River water	RWS1	A		WT	
692	Autumn 2020	River water	RWS1	A		WT	
693	Autumn 2020	River water	RWS1	A	AMP, AMC, PIP, SXT, CIP, NAL	MDR	
694	Autumn 2020	River water	RWS1	D		WT	
695	Autumn 2020	River water	RWS1	D		WT	
696	Autumn 2020	River water	RWS1	A	SXT, CIP, NAL	R	
697	Autumn 2020	River water	RWS1	B2	AMP, PIP, SXT	R	
698	Autumn 2020	River water	RWS1	D	AMP, PIP, SXT, CIP, NAL	R	
699	Autumn 2020	River water	RWS1	D		WT	
700	Autumn 2020	River water	RWS1	B1	SXT, NAL	R	
701	Autumn 2020	River water	RWS1	D		WT	
702	Autumn 2020	River water	RWS1	A	SXT	N-WT	
703	Autumn 2020	River water	RWS1	B1		WT	
704	Autumn 2020	River water	RWS1	B1		WT	
705	Autumn 2020	River water	RWS1	A		WT	
706	Autumn 2020	River water	RWS1	D		WT	
707	Autumn 2020	River water	RWS1	D		WT	
708	Autumn 2020	River water	RWS1	D		WT	
709	Autumn 2020	River water	RWS1	B1		WT	
710	Autumn 2020	River water	RWS1	A	AMP, PIP	R	
711	Autumn 2020	River water	RWS1	B1		WT	
712	Autumn 2020	River water	RWS1	D		WT	
713	Autumn 2020	River water	RWS1	B1		WT	
714	Autumn 2020	River water	RWS1	A		WT	
715	Autumn 2020	River water	RWS1	B1		WT	
716	Autumn 2020	River water	RWS1	A		WT	
717	Autumn 2020	River water	RWS1	B2	NAL	N-WT	
718	Autumn 2020	River water	RWS1	D	AMP, PIP, GM, SXT, CIP, NAL	MDR	
719	Autumn 2020	River water	RWS1	A		WT	
720	Autumn 2020	River water	RWS1	A	AMP, PIP	R	
721	Autumn 2020	River water	RWS1	B1		WT	
722	Autumn 2020	River water	RWS1	B1		WT	
723	Autumn 2020	River water	RWS1	B1		WT	
724	Autumn 2020	River water	RWS1	B1		WT	
725	Autumn 2020	River water	RWS1	D		WT	
726	Autumn 2020	River water	RWS1	B1		WT	
727	Autumn 2020	River water	RWS1	A		WT	
728	Autumn 2020	River water	RWS1	D		WT	
729	Spring 2021	River water	RWS1	B2	AMP, AMC, PIP	R	
730	Spring 2021	River water	RWS1	A	AMP, AMC, PIP	R	
731	Spring 2021	River water	RWS1	A	AMP, PIP	R	
732	Spring 2021	River water	RWS1	A	AMP, AMC, PIP	R	
733	Spring 2021	River water	RWS1	D	AMP, AMC, PIP	R	
734	Spring 2021	River water	RWS1	A	AMP, PIP	R	
735	Spring 2021	River water	RWS1	B2	AMP, PIP	R	
736	Spring 2021	River water	RWS1	B2	AMP, AMC, PIP, SXT	R	
737	Spring 2021	River water	RWS1	B2	AMP, AMC, TZP, PIP, CXM, FOX, CAZ, GN, AN	MDR	-
738	Spring 2021	River water	RWS1	B2	AMP, AMC, TZP, PIP, CXM, CAZ, FOX, AN	MDR	-
739	Spring 2021	River water	RWS1	A	AMP, PIP, CXM, CAZ, CTX, CRO, ATM, CIP, NAL	MDR	+
740	Spring 2021	River water	RWS1	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, SXT	MDR	+
742	Spring 2021	River water	RWS1	B2	AMP, AMC, PIP, SXT, NAL	R	
743	Spring 2021	River water	RWS1	B2	AMP, PIP	R	
745	Spring 2021	River water	RWS1	D	AMP, PIP, SXT	R	
746	Spring 2021	River water	RWS1	A	AMP, PIP, SXT	R	
747	Spring 2021	River water	RWS1	D	AMP, PIP	R	
748	Spring 2021	River water	RWS1	D	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, NAL	MDR	+
749	Spring 2021	River water	RWS1	B2	AMP, PIP	R	
750	Spring 2021	River water	RWS1	B2	AMP, PIP	R	
751	Spring 2021	River water	RWS1	B2	AMP, PIP	R	
752	Spring 2021	River water	RWS1	B2	AMP, AMC, PIP, GM, SXT	MDR	
753	Spring 2021	River water	RWS1	B1		WT	
754	Spring 2021	River water	RWS1	A		WT	
755	Spring 2021	River water	RWS1	B1		WT	
756	Spring 2021	River water	RWS1	B1		WT	
757	Spring 2021	River water	RWS1	B1	AMP, PIP, SXT, CIP, NAL	R	
758	Spring 2021	River water	RWS1	A		WT	
759	Spring 2021	River water	RWS1	A		WT	
760	Spring 2021	River water	RWS1	B1	AMP, PIP, SXT, CIP, NAL	R	
761	Spring 2021	River water	RWS1	A	AMP, PIP, GM	R	
762	Spring 2021	River water	RWS1	B1	AMP, PIP, SXT, CIP, NAL	R	
763	Spring 2021	River water	RWS1	A		WT	
764	Spring 2021	River water	RWS1	A	AMP, PIP, SXT, CIP, NAL	R	
765	Spring 2021	River water	RWS1	A		WT	
766	Spring 2021	River water	RWS1	A		WT	
767	Spring 2021	River water	RWS1	B1		WT	
768	Spring 2021	River water	RWS1	A		WT	
769	Spring 2021	River water	RWS1	D		WT	
772	Spring 2021	River water	RWS2	A		WT	
773	Spring 2021	River water	RWS2	A	AMP, PIP, CIP, NAL	R	
774	Spring 2021	River water	RWS2	B1		WT	

776	Spring 2021	River water	RWS2	A		WT	
777	Spring 2021	River water	RWS2	B1		WT	
778	Spring 2021	River water	RWS2	D		WT	
780	Spring 2021	River water	RWS2	D	AMP, AMC, PIP, GN, SXT, CIP, NAL	MDR	
781	Spring 2021	River water	RWS2	A	AMP, AMC, PIP, SXT, CIP, NAL	MDR	
782	Spring 2021	River water	RWS2	A	AMP, PIP, SXT	R	
783	Spring 2021	River water	RWS2	A	AMP, AMC, PIP, SXT	R	
784	Spring 2021	River water	RWS2	B1	AMP, PIP, GM	R	
785	Spring 2021	River water	RWS2	D	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, IMP, SXT, NAL	MDR	+
786	Spring 2021	River water	RWS2	B2	AMP, PIP	R	
788	Spring 2021	River water	RWS2	A	AMP, AMC, PIP, SXT	R	
789	Spring 2021	River water	RWS2	D	AMP, AMC, PIP, GN, SXT, CIP, NAL	MDR	
790	Spring 2021	River water	RWS2	D	AMP, AMC, PIP, GN, SXT, CIP, NAL	MDR	
791	Spring 2021	River water	RWS2	D	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, CIP, NAL	MDR	+
792	Spring 2021	Treated Wastewater	WWTP outlet	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, SXT	MDR	+
796	Spring 2021	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, NAL	R	
797	Spring 2021	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, SXT	R	
798	Spring 2021	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP, SXT	R	
799	Spring 2021	Treated Wastewater	WWTP outlet	B1	AMP, AMC, PIP, GN, SXT	MDR	
800	Spring 2021	Treated Wastewater	WWTP outlet	D	AMP, PIP, NAL	R	
801	Spring 2021	Treated Wastewater	WWTP outlet	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, SXT	MDR	+
802	Spring 2021	Treated Wastewater	WWTP outlet	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, SXT	MDR	+
803	Spring 2021	Treated Wastewater	WWTP outlet	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, SXT	MDR	+
804	Spring 2021	Treated Wastewater	WWTP outlet	B2	AMP, PIP	R	
805	Spring 2021	Treated Wastewater	WWTP outlet	A	AMP, AMC, PIP	R	
806	Spring 2021	Treated Wastewater	WWTP outlet	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, STX	MDR	+
807	Spring 2021	Treated Wastewater	WWTP outlet	A	AMP, PIP	R	
808	Spring 2021	Treated Wastewater	WWTP outlet	B2	AMP, AMC, PIP, GN, SXT	MDR	
809	Spring 2021	Treated Wastewater	WWTP outlet	B2	AMP, PIP, SXT	R	
810	Spring 2021	Treated Wastewater	WWTP outlet	B2	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, STX	MDR	+
811	Spring 2021	Treated Wastewater	WWTP outlet	B2	AMP, PIP	R	
812	Spring 2021	Treated Wastewater	WWTP outlet	A	AMP, PIP, SXT	R	
813	Spring 2021	Treated Wastewater	WWTP outlet	A	AMP, PIP, CIP, NAL	R	
814	Spring 2021	Treated Wastewater	WWTP outlet	B2	AMP, AMC, PIP, GN, SXT	MDR	
815	Spring 2021	Treated Wastewater	WWTP outlet	A	AMP, PIP	R	
816	Spring 2021	Treated Wastewater	WWTP outlet	B2	AMP, PIP	R	
817	Spring 2021	Treated Wastewater	WWTP outlet	D		WT	
818	Spring 2021	Treated Wastewater	WWTP outlet	D		WT	
819	Spring 2021	Treated Wastewater	WWTP outlet	D	AMP, PIP, SXT, CIP, NAL	R	
820	Spring 2021	Treated Wastewater	WWTP outlet	B1		WT	
821	Spring 2021	Treated Wastewater	WWTP outlet	B1		WT	
823	Spring 2021	Treated Wastewater	WWTP outlet	A		WT	
824	Spring 2021	Treated Wastewater	WWTP outlet	B2		WT	
825	Spring 2021	Treated Wastewater	WWTP outlet	D		WT	
826	Spring 2021	Treated Wastewater	WWTP outlet	A		WT	
827	Spring 2021	Treated Wastewater	WWTP outlet	A		WT	
828	Spring 2021	Treated Wastewater	WWTP outlet	B1	SXT	N-WT	
829	Spring 2021	Treated Wastewater	WWTP outlet	B2		WT	
830	Spring 2021	Treated Wastewater	WWTP outlet	D		WT	
831	Spring 2021	Treated Wastewater	WWTP outlet	A		WT	
832	Spring 2021	Treated Wastewater	WWTP outlet	A		WT	
833	Spring 2021	Treated Wastewater	WWTP outlet	B2	SXT	N-WT	
834	Spring 2021	Treated Wastewater	WWTP outlet	B2		WT	
835	Spring 2021	Treated Wastewater	WWTP outlet	A		WT	
836	Spring 2021	HWW	Septic tank	B2	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, CIP, NAL	MDR	+
837	Spring 2021	HWW	Septic tank	B2	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, AN, CIP, NAL	MDR	+
838	Spring 2021	HWW	Septic tank	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	+
839	Spring 2021	HWW	Septic tank	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	+
841	Spring 2021	HWW	Septic tank	B2	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, AN, CIP, NAL	MDR	+
842	Spring 2021	HWW	Septic tank	A		WT	
843	Spring 2021	HWW	Septic tank	B2		WT	
844	Spring 2021	HWW	Septic tank	B1		WT	
845	Spring 2021	HWW	Septic tank	A		WT	
846	Spring 2021	HWW	Septic tank	B2		WT	
847	Spring 2021	HWW	Septic tank	A		WT	
848	Spring 2021	HWW	Septic tank	B1		WT	
849	Spring 2021	HWW	Septic tank	B2		WT	
850	Spring 2021	HWW	Septic tank	B2		WT	
851	Spring 2021	HWW	Septic tank	B1		WT	
852	Spring 2021	HWW	Septic tank	B2	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, AN, CIP, NAL	MDR	+
853	Spring 2021	HWW	Septic tank	A		WT	
854	Spring 2021	HWW	Septic tank	B1		WT	
855	Spring 2021	HWW	Septic tank	B1		WT	
856	Spring 2021	HWW	Septic tank	B2		WT	
857	Spring 2021	HWW	Septic tank	B2		WT	
858	Spring 2021	HWW	Septic tank	A	AMP, AMC, PIP, CXM, CAZ, CTX, FOX, CRO, ATM, IMP	MDR	-
859	Spring 2021	HWW	Septic tank	B2		WT	
860	Spring 2021	HWW	Septic tank	B1		WT	
861	Spring 2021	HWW	Septic tank	B1		WT	
862	Spring 2021	HWW	Septic tank	B1		WT	
863	Spring 2021	HWW	Septic tank	B2		WT	
864	Spring 2021	HWW	Septic tank	B1		WT	
865	Spring 2021	HWW	Septic tank	A		WT	

866	Spring 2021	HWW	Septic tank	B1		WT	
867	Spring 2021	HWW	Septic tank	B2		WT	
868	Spring 2021	HWW	Septic tank	B1		WT	
869	Spring 2021	HWW	Septic tank	A		WT	
871	Spring 2021	HWW	Septic tank	B1		WT	
872	Spring 2021	HWW	Septic tank	B1		WT	
873	Spring 2021	HWW	Septic tank	B2		WT	
874	Spring 2021	HWW	Septic tank	B1		WT	
1	Summer 2019	clinical sample	urine	A	AMP, AMC	R	
2	Summer 2019	clinical sample	urine	D		S	
3	Summer 2019	clinical sample	urine	B2		S	
5	Summer 2019	clinical sample	urine	A	AMP, AMC, PIP, TCC	R	
6	Summer 2019	clinical sample	urine	A	AMP, AMC, PIP, TCC	R	
8	Summer 2019	clinical sample	urine	B2		S	
9	Summer 2019	clinical sample	urine	B2	AMP, AMC, PIP, TCC	R	
10	Summer 2019	clinical sample	blood	B2	AMP, AMC, PIP, TCC	R	
13	Summer 2019	clinical sample	urine	D	NAL	R	
17	Summer 2019	clinical sample	tissue	A	AMP, AMC	R	
22	Summer 2019	clinical sample	urine	B2	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, AN, CIP, NAL	MDR	+
27	Summer 2019	clinical sample	urine	B1	AMP, AMC, FOX	R	
28	Summer 2019	clinical sample	urine	B2	AMC	R	
29	Summer 2019	clinical sample	urine	D	AMP, AMC, PIP, SXT	R	
31	Summer 2019	clinical sample	urine	B2		S	
34	Summer 2019	clinical sample	urine	B2	AMP, AMC, CXM, FOX, MEM, IMP	MDR	
36	Summer 2019	clinical sample	urine	A	AMP, AMC, PIP, TCC, CXM, CAZ, CTX, FEP, CRO, ATM, SXT, CIP, NAL	MDR	+
40	Summer 2019	clinical sample	urine	B2	AMP, AMC, PIP, TCC	R	
43	Summer 2019	clinical sample	urine	D		S	
46	Summer 2019	clinical sample	urine	A		S	
47	Summer 2019	clinical sample	urine	B2	AMP, AMC, PIP, TCC, CXM	R	
48	Summer 2019	clinical sample	urine	B2		S	
51	Summer 2019	clinical sample	urine	B2		S	
54	Summer 2019	clinical sample	urine	B2	AMP, AMC, PIP, TZP, TCC, CXM, CAZ, CTX, FEP, CRO, ATM, GM, AN, CIP, NAL	MDR	+
58	Summer 2019	clinical sample	urine	B2	AN, NAL	R	
59	Summer 2019	clinical sample	blood	D	AMP, AMC, PIP, TZP, TCC, CXM, CAZ, CTX, FEP, CRO, ATM, SXT, NAL	MDR	+
60	Summer 2019	clinical sample	urine	B2	AMP, AMC, PIP, TCC, CXM, CTX, CRO, ATM, AN, SXT, CIP, NAL	MDR	+
61	Autumn 2019	clinical sample	urine	B2		S	
62	Autumn 2019	clinical sample	urine	B2		S	
64	Autumn 2019	clinical sample	urine	B2		S	
65	Autumn 2019	clinical sample	urine	B2	AMP, AMC, PIP, TZP, TCC, CXM, FOX, ATM (I)	MDR	
66	Autumn 2019	clinical sample	urine	B2		S	
68	Autumn 2019	clinical sample	urine	B2		S	
69	Autumn 2019	clinical sample	urine	B2		S	
70	Autumn 2019	clinical sample	urine	B2	AMP, PIP, TCC, AN	R	
77	Autumn 2019	clinical sample	blood	B2	AMP, PIP	R	
79	Autumn 2019	clinical sample	blood	B2	AMP,PIP	R	
80	Autumn 2019	clinical sample	blood	A		S	
82	Autumn 2019	clinical sample	urine	A	AMP, SXT, GM	R	
84	Autumn 2019	clinical sample	urine	D	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, FOX, ATM	MDR	+
85	Autumn 2019	clinical sample	urine	B2		S	
86	Autumn 2019	clinical sample	urine	B2		S	
87	Autumn 2019	clinical sample	urine	A		S	
90	Autumn 2019	clinical sample	urine	D	AMP, STX	R	
93	Autumn 2019	clinical sample	urine	B2		S	
97	Autumn 2019	clinical sample	blood	B2		S	
99	Autumn 2019	clinical sample	urine	A	AMP, CXM, FOX (CRO και CAZ ενδιάμεσο)	R	
110	Autumn 2019	clinical sample	urine	B2		S	
117	Autumn 2019	clinical sample	urine	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, STX	MDR	+
118	Autumn 2019	clinical sample	urine	D	AMP, AMC, PIP, CXM, CAZ, FOX, ATM (I), AN, SXT	MDR	
120	Autumn 2019	clinical sample	blood	B2		S	
122	Autumn 2019	clinical sample	urine	B2	AN, NAL	R	
123	Autumn 2019	clinical sample	urine	B2	AMP, AMC, PIP, GM	R	
129	Autumn 2019	clinical sample	urine	B2	AN	R	
130	Autumn 2019	clinical sample	urine	B2	AMC, CXM, GM, AN, STX, MEM, IMP	MDR	
133	Autumn 2019	clinical sample	blood	B2	AN	R	
135	Autumn 2019	clinical sample	urine	B2	GM, AN	R	
137	Autumn 2019	clinical sample	blood	B2		S	
139	Autumn 2019	clinical sample	blood	A		S	
145	Autumn 2019	clinical sample	urine	B2		S	
149	Autumn 2019	clinical sample	urine	A	AMP, PIP	R	
155	Autumn 2019	clinical sample	blood	A	AMP, PIP	R	
157	Winter 2020	clinical sample	urine	B2	AN	R	
165	Winter 2020	clinical sample	urine	D		S	
168	Winter 2020	clinical sample	urine	A		S	
171	Winter 2020	clinical sample	urine	A		S	
174	Winter 2020	clinical sample	urine	A		S	
175	Winter 2020	clinical sample	urine	B2	NAL	R	
181	Winter 2020	clinical sample	urine	B2	AN	R	
185	Winter 2020	clinical sample	tissue	B2	GM, AN, NAL	R	
191	Winter 2020	clinical sample	urine	D		S	
197	Winter 2020	clinical sample	urine	B2		S	
202	Winter 2020	clinical sample	urine	B2	NAL	R	

203	Winter 2020	clinical sample	blood	D	AMP, AMC, PIP, TZP, CXM, CAZ, CTX, FOX, CRO, FEP, ATM, STX, NAL	MDR	+
204	Winter 2020	clinical sample	blood	B2	AMP, AMC, PIP, AN, SXT, NAL	MDR	
210	Winter 2020	clinical sample	tissue	B2	CIP, NAL	R	
241	Summer 2020	clinical sample	urine	B2	AMP, PIP	R	
243	Summer 2020	clinical sample	urine	A		S	
245	Summer 2020	clinical sample	urine	B2	AMP, AMC, PIP, AN, STX	MDR	
246	Summer 2020	clinical sample	blood	B2	AMP, AMC, PIP	R	
249	Summer 2020	clinical sample	blood	B1	AMP, CXM, FOX	R	
253	Summer 2020	clinical sample	urine	B2		S	
254	Summer 2020	clinical sample	urine	B2	AMP, AMC, PIP	R	
255	Summer 2020	clinical sample	blood	B2		S	
259	Summer 2020	clinical sample	urine	B2		S	
261	Summer 2020	clinical sample	urine	B2		S	
264	Summer 2020	clinical sample	blood	B1	STX, CIP, NAL	R	
265	Summer 2020	clinical sample	blood	B2	AMP, AMC, PIP, GM, STX	MDR	
267	Summer 2020	clinical sample	blood	B2		S	
269	Summer 2020	clinical sample	blood	D		S	
272	Summer 2020	clinical sample	urine	D	AMP, PIP, SXT	R	
276	Summer 2020	clinical sample	urine	B2		S	
278	Summer 2020	clinical sample	urine	B2	AMP, PIP	R	
281	Summer 2020	clinical sample	urine	D	AMP, PIP, SXT	R	
284	Summer 2020	clinical sample	blood	B2		S	
286	Autumn 2020	clinical sample	urine	B2		S	
287	Autumn 2020	clinical sample	urine	B2	AMP, SXT, NAL	R	
288	Autumn 2020	clinical sample	blood	B2		S	
291	Autumn 2020	clinical sample	urine	B2		S	
292	Autumn 2020	clinical sample	tissue	B1	AMP	R	
294	Autumn 2020	clinical sample	urine	B2	AMP, PIP, CXM, CTX, CRO, ATM	R	+
296	Autumn 2020	clinical sample	urine	D	SXT	R	
297	Autumn 2020	clinical sample	urine	D	GM	R	
301	Autumn 2020	clinical sample	blood	B1		S	
303	Autumn 2020	clinical sample	urine	B2	AMP, PIP	R	
304	Autumn 2020	clinical sample	urine	B2		S	
305	Autumn 2020	clinical sample	blood	B2		S	
307	Autumn 2020	clinical sample	urine	A	AMP, PIP, GM, AN	R	
309	Autumn 2020	clinical sample	urine	D	STX	R	
311	Autumn 2020	clinical sample	urine	B2		S	
313	Autumn 2020	clinical sample	urine	D	AMP, PIP, AN, SXT	R	
314	Autumn 2020	clinical sample	blood	D	AMP, AMC, PIP	R	
318	Autumn 2020	clinical sample	urine	B2		S	
320	Autumn 2020	clinical sample	urine	B2	GM, AN	R	
324	Autumn 2020	clinical sample	urine	A		S	
325	Autumn 2020	clinical sample	blood	A	AMP, PIP, CXM, CAZ, CTX, CRO, FEP, ATM	R	+
328	Autumn 2020	clinical sample	urine	B2	AMP, PIP, IMP	R	
331	Autumn 2020	clinical sample	urine	A	STX	R	
333	Autumn 2020	clinical sample	tissue	B2	AMP, AMC, PIP, TZP, CXM, CTX, FEP, CRO, ATM, GM, CIP, NAL	MDR	+
334	Autumn 2020	clinical sample	urine	B2	CIP, NAL	R	
335	Autumn 2020	clinical sample	blood	D	NAL	R	
336	Spring 2021	clinical sample	urine	B2	AMP	R	
350	Spring 2021	clinical sample	urine	B2	AMP, PIP, CXM, CAZ, CTX, CRO, FEP, ATM, SXT, CIP, NAL	MDR	+
355	Spring 2021	clinical sample	urine	B2	AN	R	
359	Spring 2021	clinical sample	urine	B2		S	
360	Spring 2021	clinical sample	urine	A		S	
361	Spring 2021	clinical sample	urine	B2	AMP, AMC, PIP	R	
362	Spring 2021	clinical sample	urine	B2	AMP, AMC, PIP	R	
363	Spring 2021	clinical sample	urine	A		S	
364	Spring 2021	clinical sample	urine	B1	AMP, PIP, SXT	R	
365	Spring 2021	clinical sample	urine	B2		S	
366	Spring 2021	clinical sample	urine	B2		S	
367	Spring 2021	clinical sample	urine	D	AMP, AMC, PIP, CXM, AN, SXT, NAL	MDR	
368	Spring 2021	clinical sample	urine	D		S	
375	Spring 2021	clinical sample	urine	A	GM, AN	R	
378	Spring 2021	clinical sample	blood	B2	AMP, PIP, GM, AN, SXT	R	
381	Spring 2021	clinical sample	blood	D	SXT, IMP	R	
387	Spring 2021	clinical sample	blood	B2		S	
390	Spring 2021	clinical sample	urine	D	AMP, AMC, GM, AN, MEM, IMP	MDR	

Abbreviations: HWW, hospital wastewater; WWTP, wastewater treatment plant; RWS1, river water site 1; RWS2, river water site 2; AMP, ampicillin; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/ tazobactam; PIP, piperacillin; CXM, cefuroxime; CAZ, ceftazidime; CTX, cefotaxime; FOX, cefoxitin; FEP, cefepime; CRO, ceftriaxone; ATM, aztreonam; MEM, meropenem; IMP, imipenem; GM, gentamicin; AN, amikacin; SXT, sulfamethoxazole-trimethoprim; CIP, ciprofloxacin; NAL, nalidixic acid; MDR, multi-drug resistant; R, resistant'; WT, wild type; S, sensitive; DDST, double disk synergy test.

Table 3.2 Resistance rate of environmental and clinical *E. coli* isolates.

Environmental isolates	**WT**	**N-WT**	**R**	**MDR**
HWW (n=105)	54.3% (57/105)	3.8% (4/105)	7.6% (8/105)	34.3% (36/105)
WWTP (n=171)	36.8% (63/171)	3.5% (6/171)	43.3% (74/171)	16.4% (28/171)
RWS1 (n=163)	36.8% (60/163)	1.2% (2/163)	45.4% (74/163)	16.6% (27/163)
RWS2 (n=63)	36.5% (23/63)	3.1% (2/63)	42.9% (27/63)	17.5% (11/63)
Clinical isolates	**S**		**R**	**MDR**
urine (n=104):	41.3% (43/104)		45.2% (47/104)	13.5% (14/104)
blood (n=30)	43.3% (13/30)		43.3% (13/30)	13.4% (4/30)
tissue (n=5)	-		80% (4/5)	20% (1/5)

Abbreviations: RWS1, river water site 1; RWS2, river water site 2; WWTP, wastewater treatment plant; HWW, hospital wastewater; MDR, multi-drug resistant; R, resistant; N-WT, non-wild type; WT, wild type; S, sensitive

Table 3.3 The *E. coli* isolates from environmental habitats and clinical specimens that exhibit multidrug resistance					
Number of different antibiotic categories in which environmental MDR isolates presented resistance	**Number of environmental isolates obtained from each sample source**				
	HWW	**WWTP effluents**	**RWS1**	**RWS2**	**Total**
4 antibiotic categories	5	8	16	6	35
5 antibiotic categories	8	20	8	4	40
6 antibiotic categories	10		3		13
7 antibiotic categories	13			1	14
Total MDR	36	28	27	11	102
Number of different antibiotic categories in which clinical MDR isolates presented resistance	**Number of clinical isolates per specimen type**				
	Urine	**Blood**	**Tissue**		**Total**
4 antibiotic categories	2	1			3
5 antibiotic categories	5	1			6
6 antibiotic categories	5	1	1		7
7 antibiotic categories	2	1			3
Total MDR	14	4	1		19
Abbreviations: MDR, Multi-drug resistant; HWW, hospital wastewater; WWTP, wastewater treatment plant; RWS1, river water site 1; RWS2, river water site 2.					

The resistance patterns exhibited by both environmental and clinical *E. coli* isolates were classified into two categories: multiple resistant patterns (MRPs; resistance patterns to more than three antibiotic categories) and resistant patterns (RPs; resistance patterns to maximum of three different antibiotic categories).

MRPs were further separated into six sub-categories: (a) MRP1—related to ESBL production, exhibiting resistance to penicillin/inhibitor combinations (such as AMC and TZP), expanded spectrum cephalosporins (such as CTX, CRO, CAZ and FEP) with or without resistance to monobactams (ATM) and positive DDST test; (b) MPR2—related to ESBL production, showing resistance to expanded spectrum cephalosporins (such as CTX, CRO, CAZ and FEP) with or without resistance to monobactams (ATM) and positive DDST test; (c) MRP3—related to ESBL+carbapenemase production, showing resistance to expanded spectrum cephalosporins, carbapenemes (IMP and MEM) and positive DDST and CIM test; (d) MRP4—related to ESBL and AmpC production, showing resistance to cephamycins (FOX) and penicillin/inhibitor combinations (AMC and TZP) in addition to resistance to expanded spectrum cephalosporins; (e) MRP5—related to AmpC production, exhibiting resistance to cephamycins (FOX) and penicillin/inhibitor combinations (AMC and TZP) and negative DDST test and (f) other MRPs (MRP6–10) in which resistance to penicillins and to other non-β-lactam antibiotics (such as aminoglycosides, SXT and quinolones) was observed (**Table 3.4**). MRP2 and MRP1 were the most frequent MRPs among the MDR environmental and clinical isolates. Specifically, 32.3% (33/102) of the environmental MDR isolates presented an MRP2 pattern, while 28.4% (29/102) of the environmental and 36.8% (7/19) of the clinical MDR isolates presented an MRP1 pattern. Furthermore, fifty environmental and eight clinical isolates with ESBL-related MRPs presented concomitant resistance to quinolones (see **Table 3.4**).

Similarly, the RPs were further divided into five sub-categories: (a) RP1—related to ESBL production patterns with resistance to expanded spectrum cephalosporins and positive DDS test; (b) RP2—related to AmpC production with resistance to penicillins, penicillin/inhibitor combinations and cephamycin; (c) RPs3 (a–d), in which resistance to penicillins and to penicillin/inhibitor combinations with or without co-resistance to non-β-lactam antibiotics, such as quinolones, aminoglycosides and SXT, was observed; (d) RPs4 (a–g), in which resistance to penicillins with or without co-resistance to non-β-lactam antibiotics was observed and (e) RP5-6, in which only resistance to non-β-lactam antibiotics was observed (**Table 3.5**). Our results show that RP3a was the most frequent RP among 183 R environmental (30.6%; 56/183) and among 64 R clinical isolates (29.6%; 19/64). Additionally, four R environmental isolates (two from RWS1 and two from RWS2) and one R clinical isolate were found to be potential ESBL producers.

Table 3.4 Observed patterns of MDR isolates (MRP, multiple resistant patterns).			
		Environmental isolates (source)	**Clinical isolates**
MRP1: Related to ESBL production and resistance to penicillin/inhibitor combinations	PEN/ PEN–inhibitor/ ESCs + SXT	1 (WWTP)	-
	PEN/ PEN-inhibitor/ ESCs + QNs	1 (HWW)	-
	PEN/ PEN-inhibitor/ ESCs/ ATM	2 (1 RWS1, 1 RWS2)	-
	PEN/ PEN-inhibitor/ ESCs/ ATM + QNs	8 (2 HWW, 3 WWTP, 3 RWS1)	-
	PEN/ PEN-inhibitor/ ESCs/ ATM + AMG	2 (1 WWTP, 1 RWS2)	-
	PEN/ PEN-inhibitor/ ESCs/ ATM + SXT	-	1
	PEN/ PEN-inhibitor/ ESCs/ ATM + SXT + QNs	2 (1 HWW + 1 RWS2)	2
	PEN/ PEN-inhibitor/ ESCs/ ATM + AMG + QNs	2 (HWW)	3
	PEN/ PEN-inhibitor/ ESCs/ ATM + AMG + SXT + QNs	11 (HWW)	1
Total MRP1: 36		29	7
MRP2: Related to ESBL production	PEN/ ESCs/ ATM + QNs	8 (4 HWW, 3 RWS1, 1 RWS2)	-
	PEN/ ESCs/ ATM + AMG	3 (2 RWS1, 1 RWS2)	-
	PEN/ ESCs/ ATM + SXT	7 (6 WWTP, 1 RWS1)	-
	PEN/ ESCs/ ATM + SXT + QNs	2 (WWTP)	1
	PEN/ ESCs/ ATM + AMG + SXT + QNs	8 (6 HWW, 2 RWS1)	-
	PEN/ ESCs/ ATM + AMG + QNs	4 (HWW)	-
	PEN/ ESCs + SXT + QNs	1 (WWTP)	-
Total MRP2: **34**		33	1
MRP 3: Related to ESBL + carbapenemase production	PEN/ PEN-inhibitor/ ESCs/ CARB/ ATM + SXT	1 (RWS1)	-
Total MRP3: 1		1	-
MRP 4: Related to ESBL + AmpC β-lactamases production	PEN/ PEN-inhibitor/ ESCs/ FOX/ ATM	2 (1 HWW, 1 RWS1)	1
	PEN/ PEN-inhibitor/ ESCs/ FOX/ ATM + AMG + SXT	-	1
	PEN/ PEN-inhibitor/ ESCs/ FOX/ ATM + SXT + QNs	1 (HWW)	1
	PEN/ PEN-inhibitor/ ESCs/ FOX + AMG + SXT + QNs	2 (HWW)	-
Total MRP 4: 8		5	3

MRP 5: Related to AmpC β-lactamases production		PEN/ PEN-inhibitor/ FOX + AMG + QNs	1 (WWTP)	
		PEN/ PEN-inhibitor/ FOX + QNs	1 (WWTP)	
		PEN/ PEN-inhibitor/ NSCs/ FOX + AMG	2 (RWS1)	2
Total MRP5: 6			4	2
MPR 6	Susceptibility to cephalosporins Penicillinase production with resistance to other non β-lactam antibiotics	PEN/ PEN-inhibitor+ SXT+ QNs	9 (2 WWTP, 4 RWS1, 3 RWS2)	1
		Total MRP6: 10	9	1
MRP 7		PEN/ PEN-inhibitor+ AMG + SXT	10 (6 WWTP, 4 RWS1)	5
		Total MRP7: 13	10	3
MRP 8		PEN/ PEN-inhibitor +AMG + SXT+ QNs	7 (1 HWW, 1 WWTP, 2 RWS1, 3 RWS2)	2
		Total MRP8: 9	7	2
MRP 9		PEN/ PEN-inhibitor+ AMG + QNs	1 (WWTP)	-
		Total MRP9: 1	1	
MRP10		PEN+ AMG + SXT+ QNs	3 (2 WWTP, 1 RWS1)	-
		Total MRP10: 3	3	

Total MRPs: 121. Total environmental isolates with MRP: 102 and total clinical isolates with MRP: 19.

Abbreviations: MRP, multiple resistant patterns; ESBL, extended-spectrum-β-lactamase; PEN, penicillins; PEN–inhibitor, penicillin-inhibitor combinations; ESCs, Extended spectrum cephalosporins; SXT, sulfamethoxazole-trimethoprim; QNs, quinolones; ATM, aztreonam; AMG, aminoglycosides; CARB, carbapenems; FOX, cefoxitin; NSCs, narrow spectrum cephalosporins; HWW, hospital wastewater; WWTP, wastewater treatment plant; RWS1, river water site 1; RWS2, river water site 2.

Table 3.5 Observed patterns of R isolates (RP, resistant patterns)

		Environmental isolates (source)	Clinical Isolates	Total
RP1: Related to ESBL production	PEN/ ESCs	4 (2 RWS1, 2 RWS2)	-	4
	PEN/ ESCs/ ATM	-	2	2
RP2: Related to Ampc β-lactamases production	PEN/ PEN.-inhibitor/ FOX	-	1	1
RP3a	PEN/ PEN-inhibitor	56 (30 WWTP, 18 RWS1, 8 RWS2)	19	75
RP3b	PEN/ PEN-inhibitor + SXT	20 (3 HWW, 8 WWTP, 5 RWS1, 4 RWS2)	1	21
RP3c	PEN/ PEN-inhibitor + AMG	4 (3 WWTP, 1 RWS1)	2	6
RP3d	PEN/ PEN-inhibitor + QNs	18 (2 HWW, 9 WWTP, 3 RWS1, 4 RWS2)	-	18
RP4a	PEN	33 (13 WWTP, 18 RWS1, 2 RWS2)	8	41
RP4b	PEN + SXT	14 (2 WWTP, 10 RWS1, 2 RWS2)	4	18
RP4c	PEN + SXT+ QNs	11 (3 WWTP, 8 RWS1)	-	11
RP4d	PEN + QNs	10 (3 WWTP, 5 RWS1, 2 RWS2)	-	10
RP4e	PEN + AMG + QNs	-	1	1
RP4f	PEN + AMG	5 (1 WWTP, 2 RWS1, 2 RWS2)	1	5
RP4g	PEN + SXT + AMG	-	3	3
RP5 **Resistant only to non β-lactam antibiotics**	QNs	5 (3 HWW, 2 WWTP)	15	20
RP6	SXT	-	4	4
RP6a	SXT + QNs	3 (2 RWS1, 1 RWS2)	3	7

Abbreviations: RP, resistant patterns; ESBL, extended-spectrum-β-lactamase; PEN, penicillins; ESCs, extended-spectrum cephalosporins; ATM, aztreonam; PEN.-inhibitor, penicillin-inhibitor combination; FOX, cefoxitin; SXT, sulfamethoxazole-trimethoprim; AMG, aminoglycosides; QNs, quinolones; HWW, hospital wastewater; WWTP, wastewater treatment plant; RWS1, river water site 1; RWS2, river water site 2.

Furthermore, in the effort to detect any seasonal fluctuations in the R, MDR, WT/S, and N-WT populations of environmental and clinical *E. coli*, the following observations were made:

a) In the summer of 2019, the highest frequency of R isolates was observed, both among environmental (58%) and clinical (48%) isolates, as well as the highest frequency of MDR (22%) among clinical (**Figure 3.2**).

b) From the autumn of 2019 to the winter of 2019-2020, among the environmental isolates, the R populations continued to prevail over the wild-type (WT), but they showed a 3% decrease accompanied by a 12% increase in MDR (**Figure 3.2**). Regarding the clinical environment, it appeared that in the autumn of 2019, both the R and MDR populations decreased, while in the winter of 2019-20, there was an increase in R, with MDR remaining stable (**Figure 3.2**).

c) In the summer of 2020 (first wave of Covid-19), among the environmental strains, the WT was the predominant type, but during this period, the highest frequency of MDR (24%) was observed (**Figure 3.2**). During the same period, among the clinical strains, the R populations remained at the same levels as in the winter of 2019-2020, while the MDR populations decreased (**Figure 3.2**).

d) In the autumn of 2020 (first wave of Covid-19), among the environmental strains, there was a 10% increase in R isolates and a 3% decrease in MDR (**Figure 3.2**). At the same time, among the clinical strains, there was the greatest increase in R isolates but the lowest levels of MDR (**Figure 3.2**).

e) In the spring of 2021 (second wave of Covid-19), among the environmental populations, the R populations decreased, but the MDR remained stable (**Figure 3.2**). During the same period in the clinical populations, similar to the environmental, the R populations decreased, but there was a 13% increase in MDR (**Figure 3.2**).

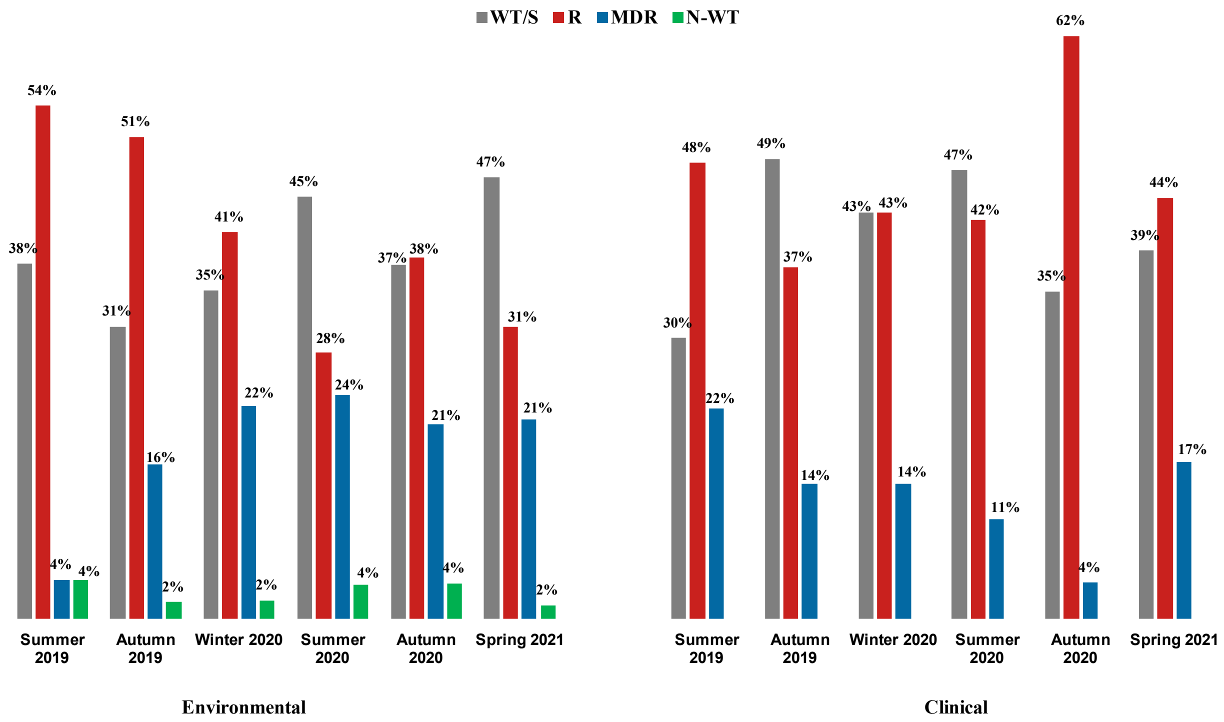


Figure 3.2 Seasonal changes in the populations of resistant (R), multidrug-resistant (MDR), wild -type/ susceptible (WT/ S) and non –wild type (N-WT) environmental and clinical E. coli *isolates*

3.3 Resistance genes detection

All of the potential β-lactamase producers (n = 84; 73 environmental and 11 clinical) were screened for β-lactamase genes. Thirty-five of the seventy- three potential β-lactamase producers from the environment were isolated from HWW, while the remaining ones were derived from the WWTP effluents (n = 14), RWS1 (n = 17) and RWS2 (n = 7). Regarding the eleven clinical potential β-lactamase producers, seven, three and one were isolated from urine, blood and tissue, respectively. The characteristics of these isolates are shown in **Table 3.6**. The blaCTX-M-group 1-type gene was detected in 52 isolates (62%; 52/84), the blaCTX-M-group 9-type gene was identified in 7 isolates (8%, 7/84), the blaTEM gene was detected in 10 isolates (13%; 11/84) and the blaSHV gene was detected in 17 isolates (20%; 17/84) (**Table 3.6**).

One isolate with an MRP3 profile was positive after the CIM test, indicating the presence of carbapenemase. Via molecular carbapenemase screening, the isolate was found to be positive for the blaOXA-48-type gene, which was identified via sequencing coding for the OXA-244 enzyme (**Table 3.6** and **Table 3.7**). In four isolates with MRP-4, the blaDHA-type, blaCMY-type, and blaFOX-type genes were detected, coding for the AmpC-type enzymes (**Table 3.6** and **Table 3.7**). Detailed data for the detection rate of the β-lactamase genes in *E. coli* isolates derived from environmental and clinical samples are summarized in **Table 3.8**. The sequencing analysis confirmed the resistance genes with an identity value of 99% to 100% (**Table 3.7, Figure 3.3**).

Finally, the sul1 gene was detected in 22/29 MDR isolates exhibiting resistance to SXT (5 clinical, 7 from HWW, 6 from WWTP, 3 from RWS1 and 1 from RWS2).

Table 3.6 Characteristics of environmental and clinical isolates harboring β-lactamase genes

Isolate ID	Type of Sample/ Sampling site	Sampling Season	Phylogenetic group	Resistance Pattern	Resistance profile		DDST	CIM test	β-lactamases	Plasmid size (MDa)
297	Treated wastewater/WWTP outlet	Summer 2019	A	AMP, AMC, PIP, CAZ, CRO, ATM, AN	MDR	MRP1	+		SHV	21.8
344	HWW/septic tank	Autumn 2019	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, CIP, NAL	MDR	MRP2	+		SHV	23.6, 15.77, 4.9
345	HWW/septic tank	Autumn 2019	A	AMP, PIP, CAZ, CTX, CRO, ATM, NAL	MDR	MRP2	+		SHV	40.2, 32.2, 9.5, 5.8
356	River water/RWS1	Autumn 2019	D	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM	MDR	MRP1	+		CTX-M group 1	6.4, 4.0
367	River water/RWS1	Autumn 2019	A	AMP, AMC, TZP, PIP, CXM, CAZ, CTX, FEP, FOX, CRO, ATM, MEM, IMP	MDR	MRP4	-	-	-	4.5
405	River water/RWS1	Winter 2020	D	AMP, AMC, PIP, CXM, CTX, CRO, ATM, CIP, NAL	MDR	MRP1	+		CTX-M group 1	32.2, 23.9
408	River water/RWS1	Winter 2020	D	AMP, AMC, TZP, PIP, TCC, CXM, CAZ, CTX, FEP, CRO, ATM, MEM, SXT	MDR	MRP3	+	+	CTX-M group 1, OXA-48	37.7, 16.4, 12.7
426	HWW/septic tank	Winter 2020	D	AMP, AMC, PIP, CXM, CAZ, CTX, CRO, ATM, CIP, NAL	MDR	MRP1	+		SHV	37.7, 30.4, 12.7, 7.0
427	HWW/septic tank	Winter 2020	B2	AMP, AMC, PIP, CXM, CAZ, CTX, CRO, ATM, AN, CIP, NAL	MDR	MRP1	+		SHV	39.1, 7.14, 6.6
431	HWW/septic tank	Winter 2020	B2	AMP, PIP, CXM, CAZ, CTX, CRO, ATM, AN, CIP, NAL	MDR	MRP2	+		SHV	39.1, 7.14, 6.6
434	HWW/septic tank	Winter 2020	B2	AMP, AMC, PIP, CAZ, CTX, CRO, ATM, CIP, NAL	MDR	MRP1	+		SHV	33.9, 17.7, 10.3, 6.4
436	HWW/septic tank	Winter 2020	B2	AMP, AMC, PIP, CXM, CAZ, CTX, CRO, ATM, AN, CIP, NAL	MDR	MRP1	+		SHV	22.3
472	River water/RWS1	Summer 2020	A	AMP, AMC, PIP, CXM, CTX, CRO, ATM, NAL	MDR	MRP1	+		CTX-M group 9, TEM	28.6
477	River water/RWS1	Summer 2020	B1	AMP, PIP, CXM, CAZ, CRO, ATM, CIP, NAL	MDR	MRP2	+		SHV	25.4, 4.0
491	Treated wastewater/WWTP outlet	Summer 2020	A	AMP, AMC, PIP, CXM, CTX, CRO, FEP, ATM, NAL	MDR	MRP1	+		CTX-M group 1	34.2
494	Treated wastewater/WWTP outlet	Summer 2020	B1	AMP, AMC, PIP, CXM, CTX, CRO, SXT	MDR	MRP1	+		TEM	21.8, 5.6
497	Treated wastewater/WWTP outlet	Summer 2020	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, NAL	MDR	MRP1	+		CTX-M group 1	22.7
506	Treated wastewater/WWTP outlet	Summer 2020	D	AMP, PIP, CXM, CAZ, CTX, FEP, ATM, SXT, NAL	MDR	MRP2	+		CTX-M group 1	31.47
510	Treated wastewater/WWTP outlet	Summer 2020	B2	AMP, PIP, CXM, CTX, CRO, SXT, CIP, NAL	MDR	MRP2	+		-	26.5
540	HWW/septic tank	Summer 2020	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	MRP1	+		CTX-M group 1	4,8, 4.0
542	HWW/septic tank	Summer 2020	A	AMP, AMC, PIP, CXM, CTX, CRO, CIP, NAL	MDR	MRP1	+		CTX-M group 1	-
543	HWW/septic tank	Summer 2020	A	AMP, AMC, PIP, CXM, CTX, CRO, FEP, ATM, GM, SXT, CIP, NAL	MDR	MRP1	+		CTX-M group 1, TEM	-

546	HWW/septic tank	Summer 2020	B2	AMP, AMC, PIP, CXM, CAZ, CTX, CRO, FOX, ATM, NAL, CIP	MDR	MRP4	+		SHV, AmpC_CMY	24.4
550	HWW/septic tank	Summer 2020	A	AMP, AMC, TZP, PIP, CXM, FOX, FEP, GM, SXT, CIP, NAL	MDR	MRP4	-		-	5.6, 4.2
552	HWW/septic tank	Summer 2020	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO ATM, GM, SXT, CIP, NAL	MDR	MRP1	+		CTX-M group 1	-
553	HWW/septic tank	Summer 2020	A	AMP, AMC, PIP, CXM, CTX, CRO, ATM, SXT, CIP, NAL	MDR	MRP1	+		CTX-M group 1	24.5, 5.8, 4.1
555	HWW/septic tank	Summer 2020	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	MRP1	+		CTX-M group 1	28.9, 10.6, 4.0
556	HWW/septic tank	Summer 2020	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, CIP, NAL	MDR	MRP2	+		SHV	48.7, 27.4, 10.8
557	HWW/septic tank	Summer 2020	A	AMP, AMC, PIP, CXM, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	MRP1	+		CTX-M group 1, TEM, SHV	22.4
558	HWW/septic tank	Summer 2020	A	AMP, AMC, PIP, CXM, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	MRP1	+		CTX-M group 1	4.8, 3.7
581	HWW/septic tank	Summer 2020	A	AMP, AMC, TZP, PIP, CXM, FOX, FEP, GM, SXT, NAL, CIP	MDR	MRP4	-		AmpC_FOX	35.6
594	River water/RWS1	Autumn 2020	B1	AMP, PIP, CXM, CAZ, CTX, CRO, ATM, CIP, NAL	MDR	MRP2	+		SHV	22.0
595	River water/RWS1	Autumn 2020	B2	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM	MDR	MRP2	+		CTX-M group 1	25.4, 4.5
598	River water/RWS1	Autumn 2020	D	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, NAL	MDR	MRP2	+		CTX-M group 1	28.6
601	River water/RWS1	Autumn 2020	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO	R	RP1	+		CTX-M group 9	28.6
602	River water/RWS1	Autumn 2020	A	AMP, PIP, CXM, CAZ, CRO, ATM	MDR	MRP2	+		-	30.3, 4.2
610	River water/RWS1	Autumn 2020	D	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, NAL	MDR	MRP2	+		CTX-M group 1	28.6
611	River water/RWS1	Autumn 2020	A	AMP, PIP, CXM, CTX, FEP, CRO	R	RP1	+		CTX-M group 9	28.6, 4.8
614	River water/RWS2	Autumn 2020	A	AMP, PIP, CXM, CTX, FEP, CRO	R	RP1	+		CTX-M group 9	30.9, 5.1
616	River water/RWS2	Autumn 2020	A	AMP, PIP, CXM, CTX, FEP, CRO	R	RP1	+		CTX-M group 9	30.9, 5.1
618	River water/RWS2	Autumn 2020	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM	MDR	MRP1	+		CTX-M group 9	29.8, 4.9
620	River water/RWS2	Autumn 2020	A	AMP, PIP, CXM, CTX, FEP, CRO, ATM, AN	MDR	MRP2	+		-	29.8, 5.1
630	River water/RWS2	Autumn 2020	A	AMP, AMC, PIP, CXM, CTX, FEP, CRO, ATM, AN	MDR	MRP1	+		-	30.9, 27, 5.2
638	Treated wastewater/WWTP outlet	Autumn 2020	D	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, SXT, NAL	MDR	MRP2	+		CTX-M group 1	33.76, 22.6
645	Treated wastewater/WWTP outlet	Autumn 2020	D	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, NAL	MDR	MRP1	+		CTX-M group 1	30.9, 27.0
673	HWW/septic tank	Autumn 2020	A	AMP, PIP, CAZ, CRO, ATM, NAL	MDR	MRP2	+		SHV	43.2, 29.3, 4.2

678	HWW/septic tank	Autumn 2020	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	MRP1	+		CTX-M group 1	31, 6.8, 3.1
680	HWW/septic tank	Autumn 2020	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	MRP1	+		CTX-M group 1	3.0, 2.0, 1.5
681	HWW/septic tank	Autumn 2020	A	AMP, PIP, CXM, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	MRP2	+		CTX-M group 1	33.4, 4.9
682	HWW/septic tank	Autumn 2020	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	MRP1	+		CTX-M group 1	32.8, 2.2, 1.7, 1.3
683	HWW/septic tank	Autumn 2020	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	MRP1	+		CTX-M group 1	55.4, 40.2, 6.9, 5.0
684	HWW/septic tank	Autumn 2020	A	AMP, PIP, CXM, CTX, CRO, ATM, GM, SXT, CIP, NAL	MDR	MRP2	+		CTX-M group 1, TEM, SHV	2.4, 1.6
685	HWW/septic tank	Autumn 2020	A	AMP, PIP, CXM, CTX, CRO, ATM, GM, SXT, CIP, NAL	MDR	MRP1	+		CTX-M group 1	-
686	HWW/septic tank	Autumn 2020	A	AMP, AMC, PIP, CXM, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	MRP1	+		CTX-M group 1	-
738	River water/RWS1	Spring 2021	B2	AMP, AMC, TZP, PIP, CXM, FOX, CAZ, GM, AN	MDR	MRP5	-		TEM	50.7
739	River water/RWS1	Spring 2021	A	AMP, PIP, CXM, CAZ, CTX, CRO, ATM, CIP, NAL	MDR	MRP2	+		SHV	25.4
740	River water/RWS1	Spring 2021	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, SXT	MDR	MRP2	+		CTX-M group 1	25.4
748	River water/RWS1	Spring 2021	D	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, NAL	MDR	MRP1	+		CTX-M group 1	32.2, 25.4
785	River water/RWS2	Spring 2021	D	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, IMP, SXT, NAL	MDR	MRP1	+	-	CTX-M group 1	48.4
791	River water/RWS2	Spring 2021	D	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, CIP, NAL	MDR	MRP2	+		CTX-M group 1	28.24, 23.62, 7.4
792	Treated wastewater/WWTP outlet	Spring 2021	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, SXT	MDR	MRP2	+		CTX-M group 1	29.53, 24.7
801	Treated wastewater/WWTP outlet	Spring 2021	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, SXT	MDR	MRP2	+		CTX-M group 1	29.53, 17.28
802	Treated wastewater/WWTP outlet	Spring 2021	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, SXT	MDR	MRP2	+		CTX-M group 1	32.3
803	Treated wastewater/WWTP outlet	Spring 2021	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, SXT	MDR	MRP2	+		CTX-M group 1	25.3, 3.4
806	Treated wastewater/WWTP outlet	Spring 2021	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, STX	MDR	MRP2	+		CTX-M group 1	25.3, 5.4, 4.5
810	Treated wastewater/WWTP outlet	Spring 2021	B2	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, STX	MDR	MRP2	+		CTX-M group 9	28.3, 18.9, 7.4

836	HWW/septic tank	Spring 2021	B2	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, CIP, NAL	MDR	MRP2	+		CTX-M group 1	-
837	HWW/septic tank	Spring 2021	B2	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, AN, CIP, NAL	MDR	MRP2	+		CTX-M group 1	-
838	HWW/septic tank	Spring 2021	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	MRP2	+		CTX-M group 1	38.6, 29.4, 26.4, 5.5
839	HWW/septic tank	Spring 2021	A	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, SXT, CIP, NAL	MDR	MRP2	+		CTX-M group 1	37.2, 6.3, 4.5
841	HWW/septic tank	Spring 2021	B2	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, GM, AN, CIP, NAL	MDR	MRP2	+		CTX-M group 1	-
852	HWW/septic tank	Spring 2021	B2	AMP, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, AN, CIP, NAL	MDR	MRP2	+		CTX-M group 1	44.2
858	HWW/septic tank	Spring 2021	A	AMP, AMC, PIP, CXM, CAZ, CTX, FOX, CRO, ATM, IMP	MDR	MRP4	-	-	AmpC_CMY	50.3, 42.8, 8.3, 6.9
22cli	clinical/urine	Summer 2019	B2	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, AN, CIP, NAL	MDR	MRP1	+		CTX-M group 1	46.0, 33.0
54cli	clinical/urine	Summer 2019	B2	AMP, AMC, PIP, TZP, TCC, CXM, CAZ, CTX, FEP, CRO, ATM, GM, AN, CIP, NAL	MDR	MRP1	+		CTX-M group 1, TEM	43, 27 .8
59cli	clinical/blood	Summer 2019	D	AMP, AMC, PIP, TZP, TCC, CXM, CAZ, CTX, FEP, CRO, ATM, SXT, NAL	MDR	MRP1	+		CTX-M group 1, TEM	35.0
60cli	clinical/urine	Summer 2019	B2	AMP, AMC, PIP, TCC, CXM, CTX, CRO, ATM, AN, SXT, CIP, NAL	MDR	MRP1	+		CTX-M group 1	34.2
84cli	clinical/urine	Autumn 2019	D	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, FOX, ATM	MDR	MRP4	+		-	41.5
117cli	clinical/urine	Autumn 2019	A	AMP, AMC, PIP, CXM, CAZ, CTX, FEP, CRO, ATM, STX	MDR	MRP1	+		CTX-M group 1, TEM	40.2, 29.2, 5.8, 5.2
203cli	clinical/blood	Winter 2020	D	AMP, AMC, PIP CXM, CAZ, CTX, FOX, CRO, ATM, STX, NAL	MDR	MRP4	+		CTX-M group 1, TEM, AmpC_DHA	44.3, 40.2
294cli	clinical/urine	Autumn 2020	B2	AMP, PIP, CXM, CTX, CRO, ATM	R	RP1	+		CTX-M group 1	29.4
325cli	clinical/blood	Winter 2021	A	AMP, PIP, CXM, CAZ, CTX, CRO, ATM	R	RP1	+		CTX-M group 1	40.2
333cli	clinical/wound	Winter 2021	B2	AMP, AMC, PIP, CXM, CTX, FEP, CRO, ATM, GM, CIP, NAL	MDR	MRP1	+		CTX-M group 1, SHV	27.9
350cli	clinical/urine	Spring 2021	B2	AMP, PIP, CXM, CAZ, CTX, CRO, FEP, ATM, SXT, CIP, NAL	MDR	MRP2	+		CTX-M group 1	50.3, 40.2, 5.3

Abbreviations: DDST, double disk synergy test; HWW, hospital wastewater; WWTP, wastewater treatment plant; RWS1, river water site 1; RWS2, river water site 2; AMP, ampicillin; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/ tazobactam; PIP, piperacillin; CXM, cefuroxime; CAZ, ceftazidime; CTX, cefotaxime; FOX, cefoxitin; FEP, cefepime; CRO, ceftriaxone; ATM, aztreonam; MEM, meropenem; IMP, imipenem; GM, gentamicin; AN, amikacin; SXT, sulfamethoxazole-trimethoprim; CIP, ciprofloxacin; NAL, nalidixic acid; MDR, multi-drug resistant; R, resistant.

Table 3.7 Sequencing results for the β-lactamase producers				
***E. coli* isolates**	**NCBI Reference Sequence**	**bla gene**	**Query cover**	**Identity value**
E. coli -22_CTX-M-group 1 (clinical/ urine)	NG_048935.1	blaCTX-M-15-like (ESBL)	99%	99.04%
E. coli -59_CTX-M-group 1 (clinical/ blood)	NG_048935.1	blaCTX-M-15-like (ESBL)	97%	99.27%
E. coli -60_CTX-M-group 1 (clinical/ urine)	NG_048935.1	blaCTX-M-15-like (ESBL)	99%	99.74%
E. coli -491_CTX-M group 1 (environmental/ WWTP)	NG_048897.1	blaCTX-M-1-like (ESBL)	96%	99.27%
E. coli -678_CTX-M-group 1 (environmental/ HWW)	NG_048935.1	blaCTX-M-15-like (ESBL)	95%	99.51%
E. coli -472_CTX-M-group 9 (environmental/ RWS1)	NG_049043.1	blaCTX-M-9-like (ESBL)	90%	99.39%
E. coli -616_CTX-M-group 9 (environmental/ RSW2)	NG_049043.1	blaCTX-M-9 (ESBL)	90%	100%
E. coli -618_CTX-M-group 9 (environmental/ RSW2)	NG_049043.1	blaCTX-M-9 (ESBL)	98%	100%
E. coli -858_CMY-2 like AmpC (environmental/ HWW)	NG_048834.1	blaCMY-4-like (AmpC type β-lactamases)	97%	99.81%
E. coli -581_FOX like AmpC (environmental/ HWW)	NG_068170.1	blaFOX-17 (AmpC type β-lactamases)	98%	100%
E. coli -408_OXA-48 like (environmental/ RWS1)	NG_049539.1	blaOXA-244 (carbapenemase_OXA-48 family class D β-lactamase)	99%	100%
E. coli -297_SHV-like (environmental/ WWTP)	NG_050087.1	blaSHV-5-like (ESBL)	97%	99.14%
E. coli -333_SHV-like (clinical/ wound)	NG_050008.1	blaSHV-13-like (ESBL)	97%	99.57%
E._coli-427_SHV-like_(environmental/HWW)	NG_050008.1	blaSHV-13-like (ESBL)	97%	99.13%
E. coli -739_SHV-like (environmetal/RWS1)	NG_050008.1	blaSHV-13 (ESBL)	97%	100%
E. coli -203_TEM-like (clinical/ blood)	NG_050186.1	blaTEM-143-like (ESBL)	99%	99.88%
E. coli -494_TEM-like (environmental/ WWTP)	NG_050239.1	blaTEM-207-like (ESBL)	99%	99.75%
E. coli -738_TEM-like (environmental/ RWS1)	NG_050186.1	blaTEM-143-like (ESBL)	99%	99.88%
Abbreviations: ESBL, extended-spectrum-β-lactamase; HWW, hospital wastewater; WWTP, wastewater treatment plant; RWS1, river water site 1; RWS2, river water site 2.				

Table 3.8 Detection rate of β-lactamase genes among clinical and environmental isolates

β-lactamase genes		Clinical isolates	Environmental isolates				Total
			HWW	WWTP effluents	RWS 1	RWS2	
ESBL genes	BlaCTX-M-group 1-type	10	22	10	8	2	52
	BlaCTX-M-group 9-type			1	3	3	7
	BlaSHV	1	12	2	3		17
	BlaTEM	4	3	2	3		12
Carbapenemase genes	BlaOXA-48-type				1		1
AmpC type genes	BlaCMY-type		2				2
	BlaFOX-type		1				1
	BlaDHA-type	1					1

Abbreviations: ESBL, extended-spectrum-β-lactamase; HWW, hospital wastewater; WWTP, wastewater treatment plant; RWS1, river water site 1; RWS2, river water site 2.

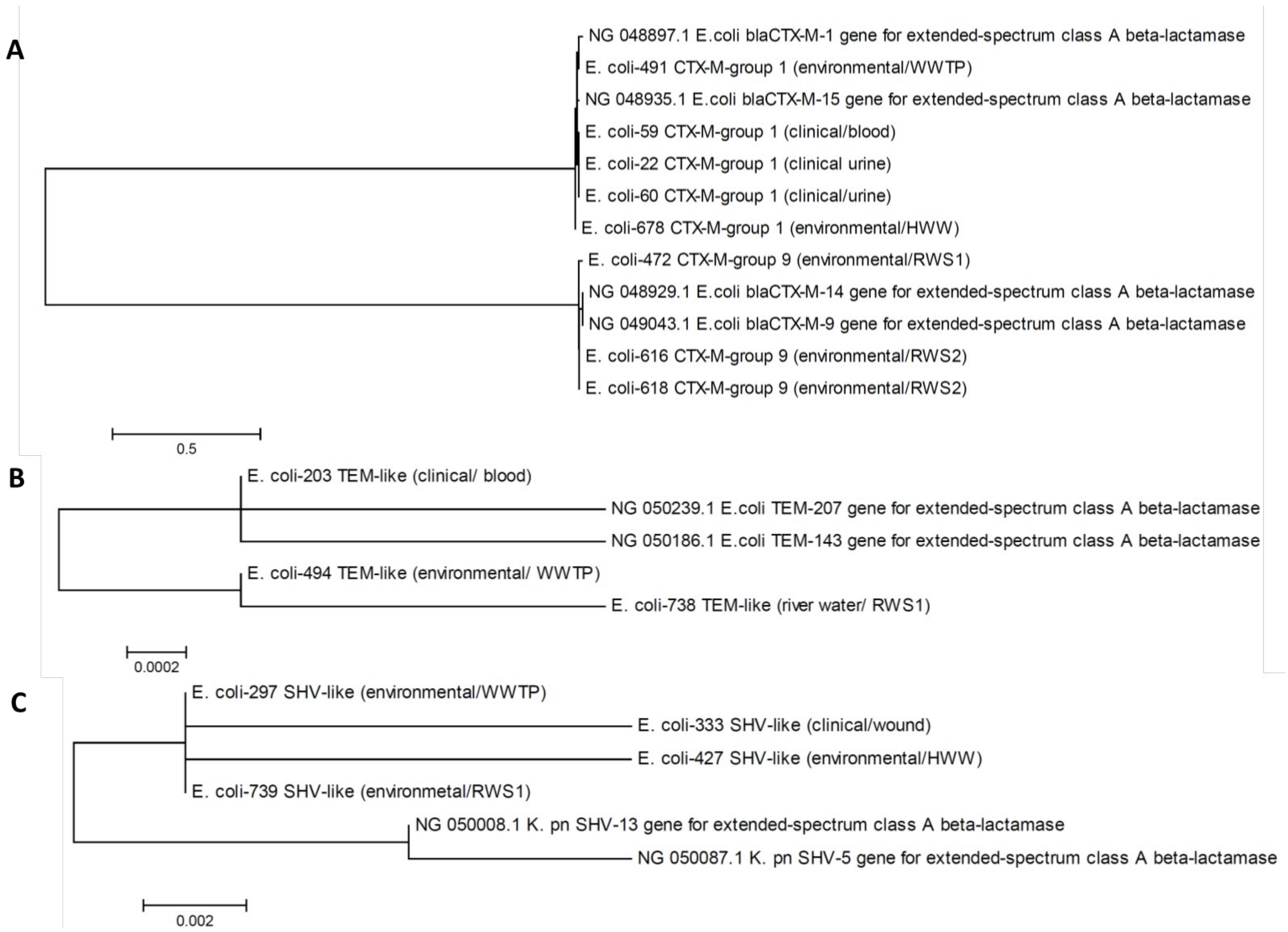


Figure 3.3 Maximum Likelihood phylogenetic trees for A) blaCTX-M-groups, B) blaTEM and C) blaSHV nucleotide sequences.

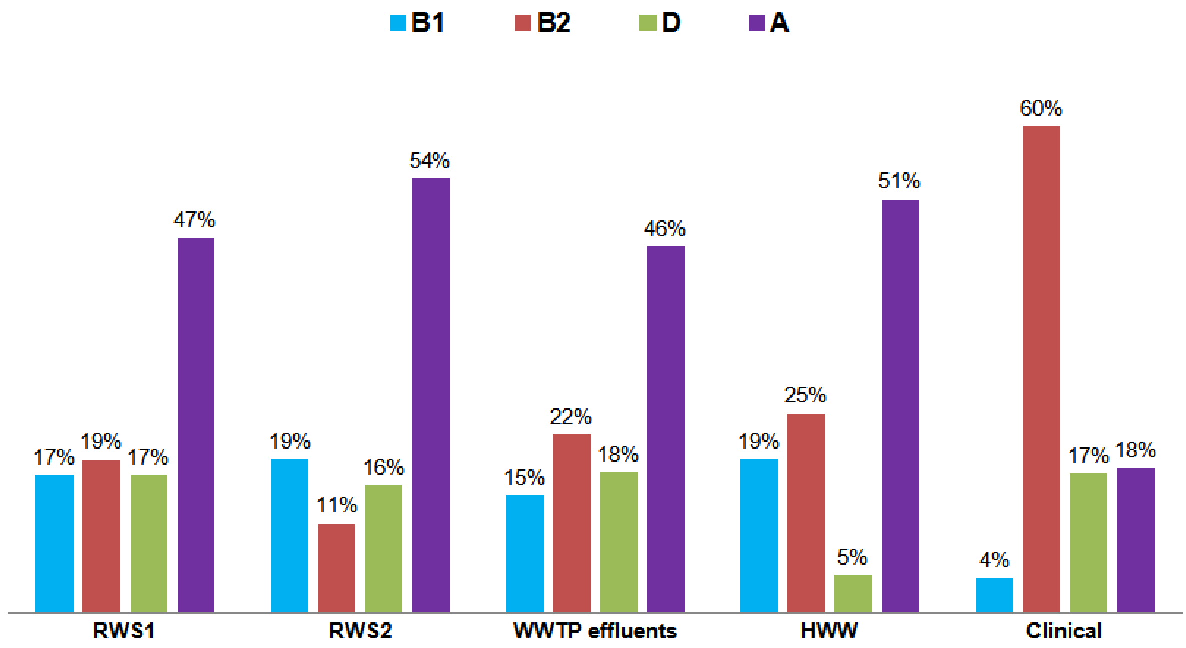
3.4 Molecular typing analysis

3.4.1 Phylogrouping typing results and statistical analysis

According to Clermont's schema *E. coli* isolates were classified into four main phylogenetic groups, A, B2, B1, and D. Based on results, There was a statistically significant correlation between the phylogenetic group and the origin of the sample [X2 (12, N = 641) = 110.63, p < 0.001)] (**Table 3.9a, b**). Group A was the predominant group (48%, 242/502) in all of the environmental sample sources, followed by B2 (20%, 102/502), B1 (17%, 85/502) and D (15%, 73/502) (**Figure 3.4a**). Moreover, the occurrence of group B2 was higher in the *E. coli* isolates from wastewater samples (WWTP effluents and HWW) compared to other environmental sources, after evaluating the adjusted ratios (**Table 3.9a, b**). In contrast to the environmental isolates, regarding the clinical isolates, group B2 was the predominant phylogenetic group (60%; 84/139), followed by A (18%, 25/139), D (17%, 24/139) and B1 (4%, 6/139) (**Figure 3.4a**). The above comparisons are in agreement with the adjusted ratios (**Table 3.9a, b**).

Furthermore, the chi-square test of independence showed that there was an association between the phylogenetic group and the resistance profiles [X2 (18, N = 641) = 184.09, p < 0.001] (**Table 3.10 a, b**). Group A was the dominant group among all of the *E. coli* populations, including MDR, R, WT and N-WT, in environmental samples, while group B2 was dominant in the clinical isolates, among all of the populations, including MDR, R and S (**Table 3.10a, b; Figure 3.4b**).

(a)



(b)

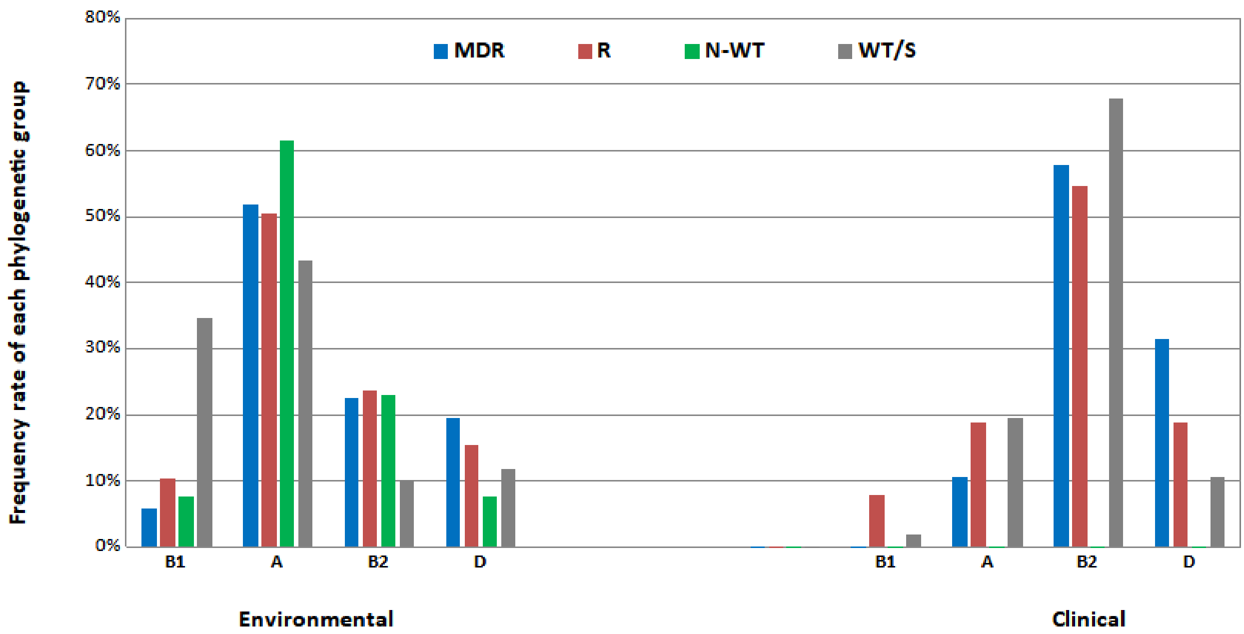


Figure 3.4 (a) The distribution of each phylogenetic group among different habitats and (b) the relationship between phylogenetic groups and resistance profiles.

[Abbreviations: RWS1, river water site 1; RWS2, river water site 2; WWTP, wastewater treatment plant; HWW, hospital wastewater; MDR, multi-drug resistant; R, resistant; N-WT, non-wild type; WT, wild type]

Table 3.9a Examination of the relationship between phylogenetic groups and origin of the sample; Sample and Group Crosstabulation							
			Groups				**Total**
			B1	**B2**	**D**	**A**	
Samples	**RWS1**	Count	28	31	28	76	163
		Expected Count	23.1	47.3	24.7	67.9	163.0
		Residual	4.9	-16.3	3.3	8.1	
		Standardized Residual	1.0	-2.4	.7	1.0	
		Adjusted Residual	1.3	-3.3	.8	1.5	
	RWS2	Count	12	7	10	34	63
		Expected Count	8.9	18.3	9.5	26.2	63.0
		Residual	3.1	-11.3	.5	7.8	
		Standardized Residual	1.0	-2.6	.2	1.5	
		Adjusted Residual	1.2	-3.3	.2	2.1	
	WWTP	Count	25	38	30	78	171
		Expected Count	24.3	49.6	25.9	71.2	171.0
		Residual	.7	-11.6	4.1	6.8	
		Standardized Residual	.1	-1.6	.8	.8	
		Adjusted Residual	.2	-2.3	1.0	1.2	
	HWW	Count	20	26	5	54	105
		Expected Count	14.9	30.5	15.9	43.7	105.0
		Residual	5.1	-4.5	-10.9	10.3	
		Standardized Residual	1.3	-.8	-2.7	1.6	
		Adjusted Residual	1.6	-1.1	-3.2	2.2	
	Clinical	Count	6	84	24	25	139
		Expected Count	19.7	40.3	21.0	57.9	139.0
		Residual	-13.7	43.7	3.0	-32.9	
		Standardized Residual	-3.1	6.9	.6	-4.3	
		Adjusted Residual	-3.8	9.2	.8	-6.4	

Total	Count	91	186	97	267	641
	Expected Count	91.0	186.0	97.0	267.0	641.0

Table 3.9b Examination of the relationship between phylogenetic groups and origin of the sample; Pearson's chi-square test results

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	110.630a	12	<.001
Likelihood Ratio	113.156	12	<.001
Linear-by-Linear Association	10.731	1	.001
N of Valid Cases	641		

a 0 cells (0.0%) have expected count less than 5. The minimum expected count is 8.94.

Table 3.10a Examination of the relationship between phylogenetic groups and resistance profile; Resistance profile and Group Crosstabulation

			Groups				Total
			B1	A	B2	D	
Environmental Resistance profile	MDR	Count	6	53	23	20	102
		Expected Count	16.4	42.5	27.7	15.4	102.0
		Residual	-10.4	10.5	-4.7	4.6	
		Standardized Residual	-2.6	1.6	-.9	1.2	
		Adjusted Residual	-3.1	2.3	-1.1	1.4	
	R	Count	19	92	43	28	182
		Expected Count	29.2	75.8	49.4	27.5	182.0
		Residual	-10.2	16.2	-6.4	.5	
		Standardized Residual	-1.9	1.9	-.9	.1	
		Adjusted Residual	-2.4	2.9	-1.3	.1	
	N-WT	Count	1	8	3	1	13
		Expected Count	2.1	5.4	3.5	2.0	13.0

		Residual	-1.1	2.6	-.5	-1.0	
		Standardized Residual	-.8	1.1	-.3	-.7	
		Adjusted Residual	-.8	1.5	-.3	-.8	
	WT	Count	71	89	21	24	205
		Expected Count	32.9	85.4	55.6	31.0	205.0
		Residual	38.1	3.6	-34.6	-7.0	
		Standardized Residual	6.6	.4	-4.6	-1.3	
		Adjusted Residual	8.8	.6	-6.6	-1.7	
Clinical Resistance profile	**S**	Count	1	11	38	6	56
		Expected Count	9.0	23.3	15.2	8.5	56.0
		Residual	-8.0	-12.3	22.8	-2.5	
		Standardized Residual	-2.7	-2.6	5.8	-.8	
		Adjusted Residual	-3.0	-3.5	7.2	-1.0	
	MDR	Count	0	2	11	6	19
		Expected Count	3.1	7.9	5.2	2.9	19.0
		Residual	-3.1	-5.9	5.8	3.1	
		Standardized Residual	-1.7	-2.1	2.6	1.8	
		Adjusted Residual	-1.9	-2.8	3.1	2.0	
	R	Count	5	12	35	12	64
		Expected Count	10.3	26.7	17.4	9.7	64.0
		Residual	-5.3	-14.7	17.6	2.3	
		Standardized Residual	-1.6	-2.8	4.2	.7	
		Adjusted Residual	-1.9	-3.9	5.2	.9	
Total		Count	103	267	174	97	641
		Expected Count	103.0	267.0	174.0	97.0	641.0

Table 3.10b Examination of the relationship between phylogenetic groups and resistance profile; Pearson's chi-square test results

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	184.099a	18	<.001
Likelihood Ratio	179.391	18	<.001
Linear-by-Linear Association	4.029	1	.045
N of Valid Cases	641		

a 5 cells (17.9%) have expected count less than 5. The minimum expected count is 1.97.

Additionally, in the effort to detect any seasonal fluctuations among the phylogenetic groups of both environmental and clinical *E. coli* isolates, it was found that:

Group A consistently remains the predominant group among environmental isolates across all seasons (**Figure 3.5**). However, in the winter of 2019-2020 and the spring of 2021 (second wave of Covid-19), the highest frequencies of group B2 were observed, at 35% and 28%, respectively (**Figure 3.5**). In the autumn of 2020 (first wave of Covid-19), an increase in group D was observed, with a frequency of 23% (**Figure 3.5**).

Regarding clinical strains, phylogenetic group B2 predominates in all seasons (**Figure 3.5**). A significant rise in group B2 was noted in the autumn of 2019 and the summer of 2020 (first wave of Covid-19), whereas group D increased in the autumn of 2020 (first wave of Covid-19) and in the spring of 2021 (second wave of Covid-19) (**Figure 3.5**).

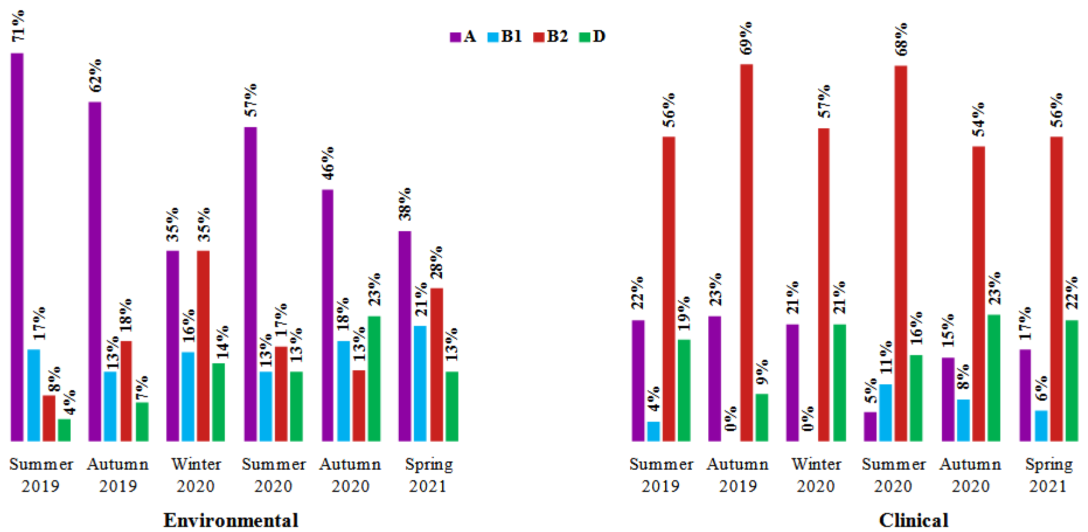


***Figure 3.5 Seasonal changes in the phylogenetics groups among environmental and clinical E. coli* isolates**

3.4.2. Pulsed field gel electrophoresis (PFGE) results

PFGE was applied to 51 representative MDR isolates derived from different environments (6 clinical isolates, 17 from HWW, 13 from WWTP effluents, 8 from RSW1, and 7 from RSW2), in order to group them into clusters based on the percentage similarity of their PFGE patterns. The results revealed considerable heterogeneity (**Figure 3.6**), even among strains such as those from the clinical setting and HWW, which have a very close epidemiological relationship. However, as illustrated in the **Figure 3.6**, certain isolates, that exhibited the same resistance pattern and carried the same resistance gene, such as 683, 684, 681 (derived for HWW) and the clinical isolate 350 (see Table 3.1), displayed the same PFGE pattern.

Among the clinical isolates, substantial heterogeneity was also observed, which is expected, given that most the clinical strains were not isolated from hospitalized patients (i.e., they are not related to nosocomial infections) but from emergency cases.

In conclusion, PFGE analysis revealed diverse genetic fingerprints and thus did not provide additional information on the molecular classification of the *E. coli* isolates.

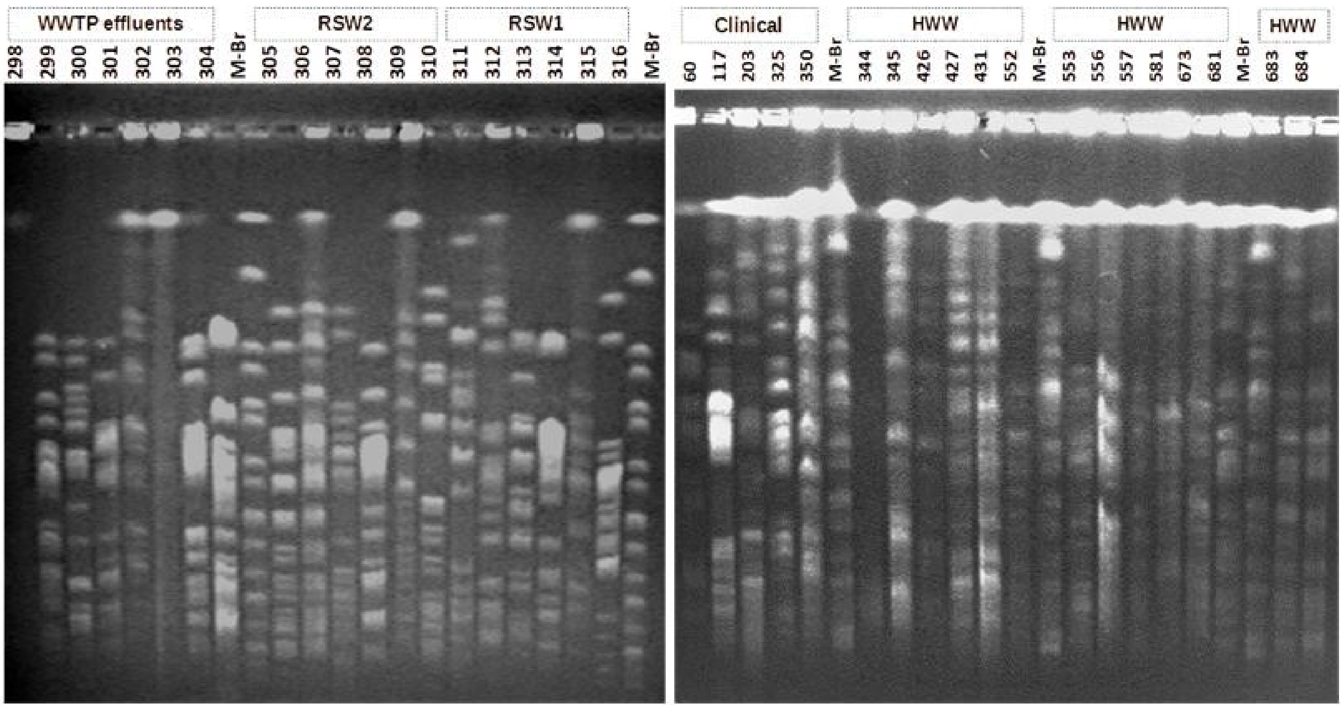


Figure 3.6 PFGE analysis; Diverse PFGE patterns of E. coli isolated from clinical and environmental samples.

[Abbreviations: HWW, hospital wastewater; WWTP, wastewater treatment plant; RWS1, river water site 1; RWS2, river water site 2]

3.4.3 Plasmid typing resutls

According to the results of plasmid typing, plasmids with molecular size larger than 20 MDa were detected in sixty nine out of the eighty four β-lactamase producers that were subjected to this analysis. Eight isolates harbored small plasmids with a molecular size of less than 8 MDa, while no plasmids were detected in the remaining seven strains. The **Table 3.6** presents the results of plasmid typing (plasmid patterns) as well as and other characteristics (such as type of sample, resistance pattern, resistance gene, phylogenetic group) of the eighty four isolates that were analyzed.

Although the plasmid profiles showed heterogeneity, some strains exhibited similar or identical patterns, as with isolates 601, 611, 614, 616, 618, 620 shown in the **Figure 3.7**, which derived from river water (RWS1 and RWS2) and carry the ESBL gene blaCTX-M-group-9 (see **Table 3.6**).

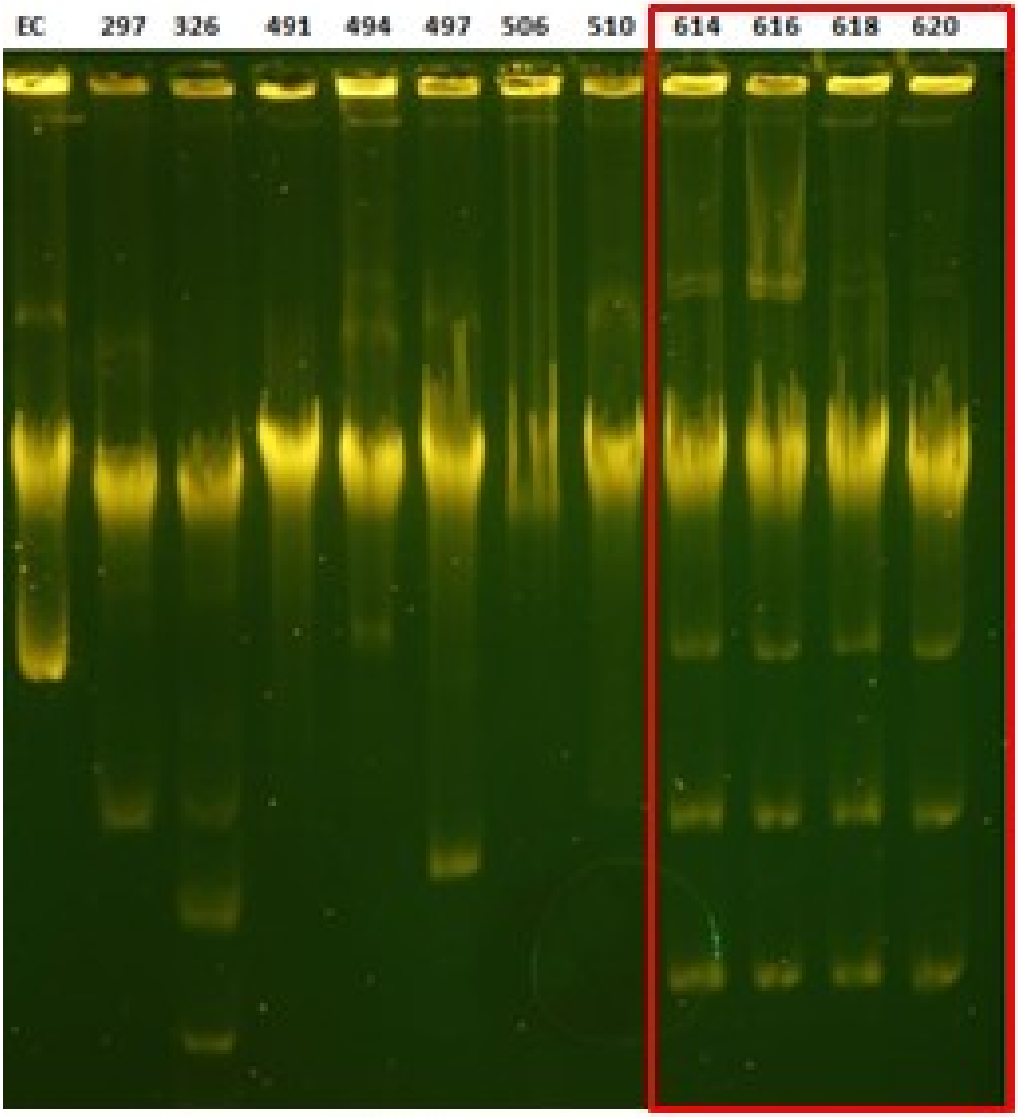


Figure 3.7 Electrophoresis of plasmid DNA.

3.5 Results of resistance transfer frequency

Out of thirty three (27 environmental and 6 clinical) in only thirteen β-lactamase producing *E. coli* isolates, the conjugation experiments were successful in transferring β-lactam resistance at a high rate (**Table 3.11**). It is likely that the remaining 20 strains possessed non-conjugative plasmids or that the β-lactamase genes were chromosomally located. The majority of those thirteen paternal strains (donors) were characterized as MDR (84.3%; 11/13) and the most frequent resistance was to CTX and FEP (84.3%; 11/13) followed by the resistance to ATM (76.9%; 10/13). According to results of antimicrobial susceptibility test which was performed in all plasmid recipients isolates (transconjugants), their resistance patterns were similar or identical with their corresponding donors (**Table 3.11**). For β-lactams antibiotics the transmitted resistance reached 100% for CTX and ATM, and 83.3%, 81.8%, 50% and 25% for CAZ, FEP, FOX and AMC respectively (**Table 3.11**). For non β-lactams antibiotics, resistance to NAL was transferred to seven of the thirteen transconjugant clones (54%, 7/13), to TOB and SXT in three clones (23%, 3/13), and to CIP in one clone (7.6%, 1/13) (**Table 3.11**).

The plasmid sizes of the transconjugant clones ranged between 21-48 MDa, and most showed plasmid patterns similar to those of their corresponding parental strains (**Table 3.11**). PCR was used to detect β-lactamase genes in the transconjugant clones. According to PCR results, most of the transconjugant clones contained the same genes as their corresponding parental strains (**Table 3.11**). Of the thirteen parental strains capable of transferring plasmids, seven carried the blaCTX-M-group-1 gene, three carried the blaCTX-M-group-9 gene, and three carried the blaSHV-type gene. Additionally, four parental strains, 203cli, 408, 472, and 546, carried more than one β-lactamase gene (**Table 3.11**). Specifically, in parental clone 203cli, the β-lactamase genes blaCTX-M-group 1, blaTEM-type, and blaDHA-type were detected, in parental clone 408 the genes blaCTX-M group 1 and blaOXA-48-type, in parental clone 472 the genes blaCTX-M group 9 and blaTEM-type, and in clone 546 the genes blaSHV-type and blaCMY-type (**Table 3.11**). However, in the transconjugant clones derived from parental strains 203cli, 408, 472, and 546, which encoded more than one β-lactamase, only the blaDHA-type (blaDHA-1), blaCTX-M group 1, blaCTX-M group 9, and blaSHV genes, respectively, were transferred. No clones were selected that transferred the blaCTX-M group 1 + blaTEM, blaOXA-48-type, blaTEM-type, and blaCMY-type genes. This explains the differences observed in resistance patterns between these specific transconjugant clones and their corresponding parental strains (**Table 3.11**) and leads to the conclusion that the genes are located on different genetic units. The inability to transfer the remaining genes may be due either to the very low frequency of plasmid transfer, which is undetectable under the applied laboratory conditions, or to the chromosomal location of the genes. Additionally, the resistance to non-β-lactam antibiotics, such as NAL, CIP, TOB, and SXT, observed in some transconjugant clones, suggests that the transferred plasmids carried multiple resistance genes and not only β-lactamase genes.

Table 3.11 Comparison of resistance traits between transconjugant and parental clones

Isolate	Type of Sample/ Sampling site	Phylogenetic group	Resistance Pattern	DDS test	CIM test	β-lactamases	Plasmid profile (MDa)	Transfer frequency	Incompatibility group	Other resistance determinants
203cli	blood/urine	D	AMP, AMC, PIP, CAZ, CTX, CRO, FEP, ATM, FOX, IPM, MEM, SXT, NAL	-	-	CTX-M group 1, TEM, DHA-1	44.3, 40.2	$0.087*10^{3}$	IncFII	qnrB4, sul1, dfrA17
trc203cli			AMP, AMC, PIP, CAZ, CTX, ATM, FOX, IPM, SXT, NAL	-	-	DHA-1	40.2		IncFII	qnrB4, sul1, dfrA17
294cli	urine/clinical	B2	AMP, PIP, CTX, CRO, FEP, ATM, SXT, NAL	+	NT*	CTX-M group 1	29.4	$0.096*10^{3}$	ND**	ND
trc294cli			AMP, PIP, CTX, CRO, FEP, ATM, SXT, NAL	+	NT	CTX-M group 1	29.4		ND	ND
297	wastewater/ WWTP	A	AMP, PIP, CAZ, ATM, NAL	+	NT	SHV-12	21.8	$0.4*10^{4}$	IncX3	qnrS1, aac(6')-Ib3
trc297			AMP, PIP, CAZ, ATM, NAL	+	NT	SHV-12	21.8		IncX3	qnrS1, aac(6')-Ib3
408	river water/ RWS1	D	AMP, AMC, PIP, CTX, CRO, FEP, ATM, MEM, SXT	+	+	CTX-M group 1, OXA-48	37.7, 16.4, 12.7	$0.098*10^{3}$	ND	ND
trc408			AMP, PIP, CTX, CRO, ATM	+	-	CTX-M group 1	37.7		ND	ND
472	river water/ RWS1	A	AMP, AMC, PIP, CTX, CRO, FEP, NAL	+	NT	CTX-M group 9, TEM	28.6	$0.12*10^{4}$	ND	ND
trc472			AMP, PIP, CTX, CRO, FEP, NAL	+	NT	CTX-M group 9	28.6		ND	ND
506	wastewater/ WWTP	D	AMP, PIP, CTX, CRO, FEP, ATM, SXT, NAL	+	NT	CTX-M group 1	31.5	$2.25*10^{2}$	ND	ND
trc506			AMP, PIP, CTX, CRO, FEP, ATM	+	NT	CTX-M group 1	31.5		ND	ND

546	HWW/ septic tank	B2	AMP, AMC, PIP, CAZ, CTX, CRO, FEP, ATM, FOX, NAL, CIP	+	NT	SHV, CMY	24.4	$0.29*10^{-2}$	ND	ND
trc546			AMP, PIP, CAZ, CTX, CRO, FEP, ATM, NAL, CIP	+	NT	SHV	24.4		ND	ND
556	HWW/ septic tank	A	AMP, PIP, CAZ, ATM, NAL	+	NT	SHV	48.7, 27.4, 10.8	$0.428*10^{-2}$	ND	ND
trc556			AMP, PIP, CAZ, ATM, NAL	+	NT	SHV	48.7, 27.4		ND	ND
601	river water/ RWS1	A	AMP, PIP, CTX, CRO, FEP, TOB	+	NT	CTX-M group 9	28.6, 5.4	$0.83*10^{-2}$	ND	ND
trc601			AMP, PIP, CTX, CRO, FEP, TOB	+	NT	CTX-M group 9	28.6		ND	ND
610	river water/ RWS1	D	AMP, PIP, CAZ, CTX, CRO, FEP, ATM, GM, TOB, SXT, NAL	+	NT	CTX-M group 1	28.6	$0.11*10^{-6}$	ND	ND
trc610			AMP, PIP, CTX, CRO, FEP, ATM, TOB	+	NT	CTX-M group 1	28.6		ND	ND
618	river water/ RWS2	A	AMP, PIP, CTX, CRO, FEP, ATM, TOB	+	NT	CTX-M group 9 (CTX-M-14)	29.8, 4.9	$0.875*10^{-2}$	IncFII	mph(A), cmlA1, aac(6')-Ib3, sul1, qacE
trc618			AMP, PIP, CTX, CRO, FEP, ATM, TOB	+	NT	CTX-M group 9 (CTX-M-14)	29.8		IncFII	mph(A), cmlA1, aac(6')-Ib3, sul1, qacE
638	wastewater/ WWTP	D	AMP, PIP, CAZ, CTX, CRO, FEP, ATM, SXT, NAL	+	NT	CTX-M group 1	33.8, 22.6	$0.16*10^{-4}$	ND	ND
trc638			AMP, PIP, CAZ, CTX, CRO, FEP, ATM, SXT	+	NT	CTX-M group 1	33.8		ND	ND
791	river water/ RWS2	D	AMP, PIP, CTX, CRO, FEP, NAL	+	NT	CTX-M group 1	28.3, 23.6, 7.4, 4.5	$0.58*10^{-3}$	ND	ND
trc791			AMP, PIP, CTX, CRO, FEP, NAL	+	NT	CTX-M group 1	28.3		ND	ND

Trc: transconjugant clone

*NT: not tested,

**ND: not determined

Abbreviations: AMP, ampicillin; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/ tazobactam; PIP, piperacillin; CXM, cefuroxime; CAZ, ceftazidime; CTX, cefotaxime; FOX, cefoxitin; FEP, cefepime; CRO, ceftriaxone; ATM, aztreonam; MEM, meropenem; IMP, imipenem; GM, gentamicin; AN, amikacin; TOB, Tobramycin; SXT, sulfamethoxazole-trimethoprim; CIP, ciprofloxacin; NAL, nalidixic acid, DDST, double disk synergy test; CIM, carbapenem inactivation method;

3.6 NGS plasmid analysis:sequencing, assembly, annotation

Sequencing of β-lactamase gene- carrying plasmids ptrc203cli, ptrc297, ptrc618 which harbored the genes blaDHA-type, blaSHV-type and blaCTX-M-group-1, respectively was performed using Oxford Nanopore Technology (ONT). Sequencing and the resulting reads are then subjected to quality filtering, assembly, and annotation using the Nanopore data analysis pipeline developed by Eurofins. The draft sequence of those plasmids was used for characterization of the β-lactamase genetic environment.

a) Genomic features of the plasmid ptrc203cli

Plasmid analysis reveal that the plasmid ptrc203cli, which was isolated from the transconjugant clone trc203cli, had a size of 81.582 bp and, according to the results of PlasmidFinder, belonged to the IncFII incompatibility group (**Figure 3.8a**). ResFinder results indicated that plasmid ptrc203cli carried multiple ARGs conferring resistance to extended- spectrum cephalosporins (blaDHA-1), sulfonamides (sul1), trimethoprim (trimethoprim-resistant dihydrofolate reductase gene, dfrA17) and reduced susceptibility to fluoroquinolones (quinolone resistance pentapeptide repeat gene, qnrB4) **Figure 3.9a.** The **Table 3.12** presents the results of ResFinder.

According to the analysis through Proskee, upstream of the blaDHA-1 (ampC) were also detected the transcriptional activator ampR, which regulate ampC β-lactamase expression. The resistance genes were flanked by transposable elements, specifically by insertion sequences (IS elements). Both the resistance genes and the IS elements were located in a specific section of the plasmid, approximately 19.500 bp in size, forming the multidrug resistance (MDR) region. More specifically, within this MDR region, the blaDHA-1 gene, the ampR gene, the qnrB4 gene, and the sul1 gene were located within a region flanked by IS26 sequences, forming the transposable genetic unit IS26-qnrB4...//..blaDHA-1-ampR-sul1-IS26-IS1R (**Figure 3.9a**).

According to the results of BLAST and BRIG analyses, the ptrc203cli plasmid showed a total coverage of 95% with the previously described plasmids pUB_DHA-1 (GenBank accession no. MK048477.1) and p3-S1-IND-02-A (GenBank accession no. CP145649.1), with the identity in the covered regions reaching 99.95% and 100%, respectively (**Figure 3.10a**). These two reference plasmids, like ptrc203cli, had been detected in *E. coli* strains isolated from human biological samples, specifically from feces and urine

b) Genomic features of the plasmid ptrc297

Plasmid analysis shown that plasmid ptrc297, which was isolated from the transconjugant clone trc297cli, had a size of 46.338 bp and, according to the results of PlasmidFinder, was classified within the IncX3 incompatibility group (**Figure 3.8b**). ResFinder results indicated that plasmid ptrc297 carried ARGs conferring resistance to extended- spectrum cephalosporins (blaSHV-12) and reduced susceptibility to quinolones (qnrS1). The **Table 3.12** presents the results of ResFinder for the plasmid ptrc297.

The ARGs and the IS elements were located on a ~10000 bp fragment of the ptrc297 plasmid, forming the resistance region. Specifically, this resistance region of ptrc297 was enclosed by the transposable elements ISKpn19 and IS26, within which the qnrS1 and blaSHV-12 genes were located, forming the genetic region ISKpn19 - qnrS1 - IS26 - blaSHV-12 - IS26 (**Figure 3.9b**). According to the BLAST and BRIG analyses, the ptrc297 plasmid had a coverage rate of 52%, 50%, and 49%, respectively, with the already characterized plasmids pCF12 (GenBank accession no. MT720906.1), pTKEC21-17 (GenBank accession no. CP092451.1), and pEC-147 (GenBank accession no. KX618702.1), while the identity in the regions where coverage was observed reached 99.9% in all three cases. Regarding the origin of the reference plasmids, the pCF12 plasmid was isolated from *Citrobacter freundii*, while the pTKEC21-17 and pEC-147 plasmids were isolated from *E. coli* isolates, which had been recovered from sewage and poultry feces, respectively (**Figure 3.10b**).

c) Genomic features of the plasmid ptrc618

Plasmid analysis reveal that the plasmid ptrc618, which was isolated from the transconjugant clone trc618, had a size of 104.665 bp and, and based on the PlasmidFinder results it belongs to incompatibility group IncFII (**Figure 3.8c**). ResFinder results indicated that plasmid ptrc618 harbored multiple ARGs conferring resistance to extended- spectrum cephalosporins (blaCTX-M-14), macrolides (mphA), aminoglycosides [aac (6')-Ib3] and chloramphenicol (cmlA1). The **Table 3.12** presents the results of ResFinder for the plasmid ptrc618.

The analysis through Proskee showed that the resistance genes and the adjacent transposable IS elements were located in a region approximately 25.000 bp in size, forming the MDR region of the plasmid. More specifically, within the MDR region were located: (a) the operon mphA-mrx(A)-mphR(A), which was linked to macrolide resistance, was bracketed by the elements IS26 (downstream) and IS6100 (upstream), (b) the integron type 1, which contained the integrase gene int1 and the genes aac(6')-Ib3, cmlA1, deltaqacE (antiseptic-resistance gene), sul1, and (c) the gene blaCTX-M-14, flanked by the transposable elements ISEcp1-IS903B (upstream) and ISEcp1-IS26 (downstream),

forming the transposable genetic structure IS26-- mphA-mrx(A)-mphR(A) -- IS6100…int1-- aac(6')-Ib3 -- cmlA1 -- deltaqacE-- sul1—ISpsy43-- ISEcp1-IS903B -- blaCTX-M-14-- ISEcp1-IS26 (**Figure 3.9c**).

The results from the analyses using the software BLAST and BRIG revealed that the plasmid ptrc618 had a total coverage of 93% and 88%, respectively, with the previously characterized plasmids pCTXM14_005215 (GenBank accession no. CP092974.1) and pEC22-3 (GenBank accession no. CP060894.1). The similarity percentage for the regions covered was 99.9% in both cases. These two reference plasmids were isolated from *E. coli* strains detected in human biological samples (rectal swabs and sputum) (**Figure 3.10c**).

Both IncX3 and IncFII plasmids are conjugative, meaning they can spread resistance genes through horizontal gene transfer, but the range of species they can transfer to differs, with IncX3 having a broader reach. IncX3 plasmids can replicate in various Gram-negative bacteria, particularly within the Enterobacteriaceae family. Their host range is broader than some narrow host range plasmids but not as extensive as those with very broad host ranges. On the other hand, IncFII plasmids are primarily restricted to Enterobacteriaceae, such as *E. coli*, Salmonella spp, and Klebsiella spp. They do not typically replicate in as broad a range of hosts as IncX3 plasmids.

Table 3.12 ResFinder results for plasmids ptrc203cli, ptrc297 and ptrc618								
plasmid	Resistance gene	Identity	Alignment Length/ Gene Length	Coverage	Position in reference	Position in contig	Phenotype	Accession no.
ptrc_203cli	blaDHA-1	99.91%	1140/1140	100	1….1140	3239..4378	Amoxicillin, Amoxicillin+Clavulanic acid, Ampicillin, Ampicillin+Clavulanic acid, Cefotaxime, Cefoxitin, Ceftazidime, Piperacillin, Piperacillin+Tazobactam, Ticarcillin, Ticarcillin+Clavulanic acid	Y16410
	qnrB4	100%	645/645	100.0	1..645	80053..80697	Ciprofloxacin	DQ303921
	sul1	100%	840/840	100.0	1..840	5955..6794	Sulfamethoxazole	U12338
	dfrA17	100%	474/474	100.0	1..474	74645..75118	Trimethoprim	FJ460238
ptrc_297	blaSHV-12	100%	861/861	100	1..…861	15076..15936	Amoxicillin, Ampicillin, Aztreonam, Cefepime, Cefotaxime, Ceftazidime, Ceftriaxone, Piperacillin, Ticarcillin	KF976405
	qnrS1	100%	657/657	100	1….657	13074..13730	Ciprofloxacin	AB187515
ptrc_618	aac(6')-Ib3	100%	555/555	100	1….555	86435..86989	Amikacin, Tobramycin	X60321
	blaCTX-M-14	100%	876/876	100	1….876	92310..93185	Amoxicillin, Ampicillin, Aztreonam, Cefepime, Cefotaxime, Ceftazidime, Ceftriaxone, Piperacillin, Ticarcillin	AF252622
	mph(A)	100%	906/906	100	1….906	80505..81410	Erythromycin, Azithromycin, Spiramycin, Telithromycin	D16251
	cmlA1	99.92%	1260/1260	100	1….1260	87256..88515	Chloramphenicol	AB212941

(a)

```
Plasmid      Identity  Query / Template length    Contig                 Position in contig    Note    Accession number
*************************************************************************************************************************
IncFII            100  261 / 261                  11108976295_trc203cli  70809..71069          -       AY458016
=========================================================================================================================



Extended Output:

# IncFII_AY458016
template:       CACACCATCCTGCACTTACAATGCGCAGAAGGAGCGAGCACAGAAAGAAGTCTTGAACTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
query:          CACACCATCCTGCACTTACAATGCGCAGAAGGAGCGAGCACAGAAAGAAGTCTTGAACTT

template:       TTCCGGGCATATAACTATACTCCCCGCATAGCTGAATTGTTGGCTATACGGTTTAAGTGG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
query:          TTCCGGGCATATAACTATACTCCCCGCATAGCTGAATTGTTGGCTATACGGTTTAAGTGG

template:       GCCCCGGTAATCTTTTCGTACTCGCCAAAGTTGAAGAAGATTATCGGGGTTTTTGCTTTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
query:          GCCCCGGTAATCTTTTCGTACTCGCCAAAGTTGAAGAAGATTATCGGGGTTTTTGCTTTT

template:       CTGGCTCCTGTAAATCCACATCAGAACCAGTTCCTTGCCACCTTACGGCGTGGCCAGCCA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
query:          CTGGCTCCTGTAAATCCACATCAGAACCAGTTCCTTGCCACCTTACGGCGTGGCCAGCCA

template:       CAAAATTCCTTAAACGATCAG
                |||||||||||||||||||||
query:          CAAAATTCCTTAAACGATCAG
```

(b)

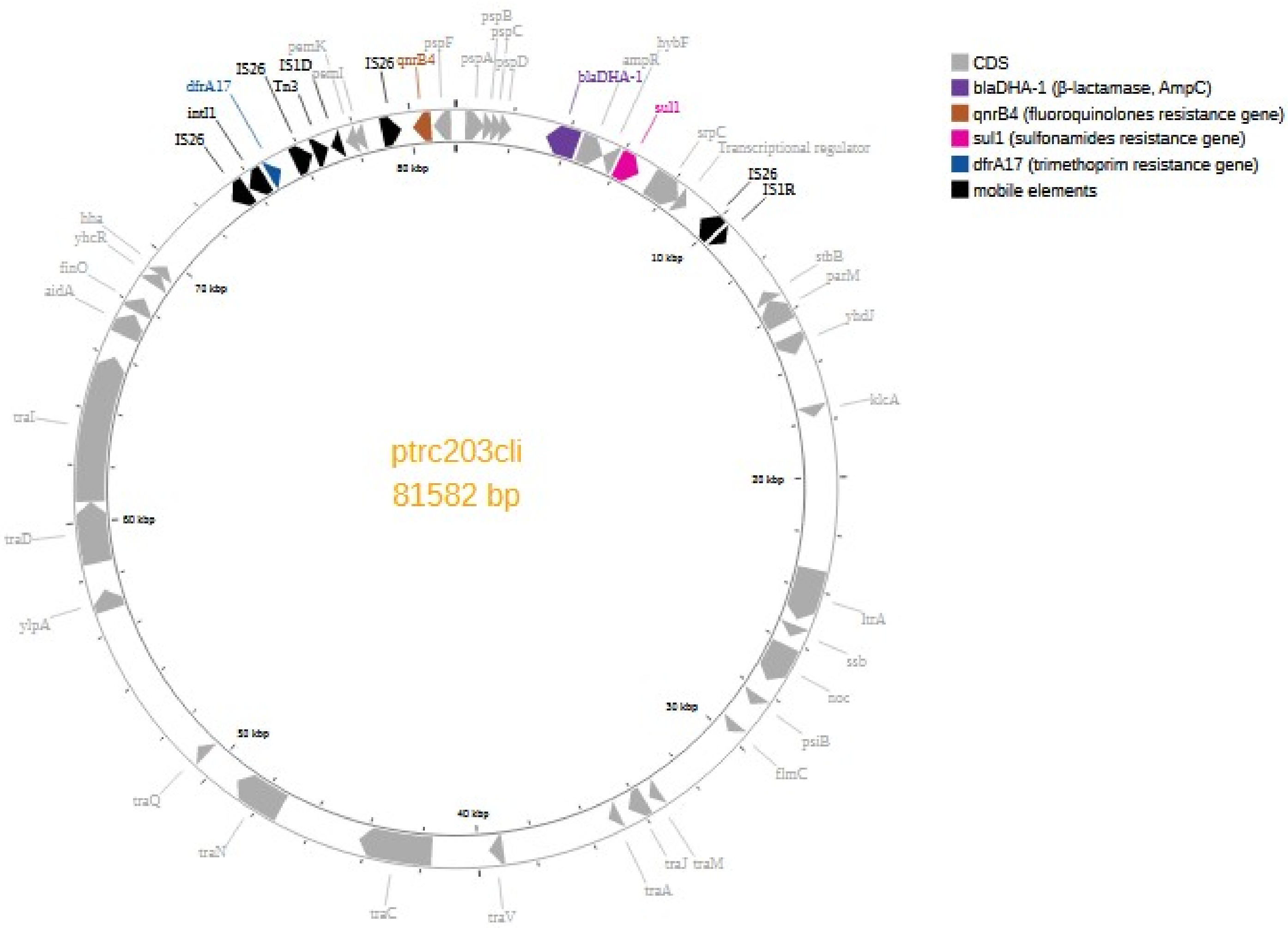
```
*****************************************************************************************************************
Plasmid        Identity  Query / Template length    Contig                Position in contig   Note     Accession number
*****************************************************************************************************************

IncX3          100       374 / 374                  11108795091_trc297_2  23166..23539         -        JN247852
IncX3           99.73    374 / 374                  11108795091_trc297_2  219..592             -        JN247852
=================================================================================================================



Extended Output:

# IncX3_JN247852
template:       ATGCGGTTGTTGCTATCTTTAGATATGAAGATCCTCAGATCTTCATATCTAAAGGTGAGA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
query:          ATGCGGTTGTTGCTATCTTTAGATATGAAGATCCTCAGATCTTCATATCTAAAGGTGAGA

template:       GGTTTTTTAATTAAAGGTTGTATTGTTGTCTTGAATTACAACCTTTGTGGGGTTATGATT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
query:          GGTTTTTTAATTAAAGGTTGTATTGTTGTCTTGAATTACAACCTTTGTGGGGTTATGATT

template:       TGCCTACATAGGAAAGGTTATATGAGGCTTATCGTGAAGACAGTAACGGGATTAACGAAA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
query:          TGCCTACATAGGAAAGGTTATATGAGGCTTATCGTGAAGACAGTAACGGGATTAACGAAA

template:       GTTAGACATAGAAATGAAGTTGGGGTAACTCTTGCATCCCTTTCCCTTTCAGCAAAAAGA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
query:          GTTAGACATAGAAATGAAGTTGGGGTAACTCTTGCATCCCTTTCCCTTTCAGCAAAAAGA

template:       GTGCTTTTTCTGGCTCTTTGCCAGATTGATACAAAGGAAATGTTAGATGATGATATTTTG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
query:          GTGCTTTTTCTGGCTCTTTGCCAGATTGATACAAAGGAAATGTTAGATGATGATATTTTG

template:       GAGGTTGATGCTGACTTTTTTTCAAAAGCTACTTCTTTAGATAAATATGCCTCTTATGCA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
query:          GAGGTTGATGCTGACTTTTTTTCAAAAGCTACTTCTTTAGATAAATATGCCTCTTATGCA

template:       GCTCTGAAAGAGGG
                ||||||||||||||
query:          GCTCTGAAAGAGGG
```

(c)

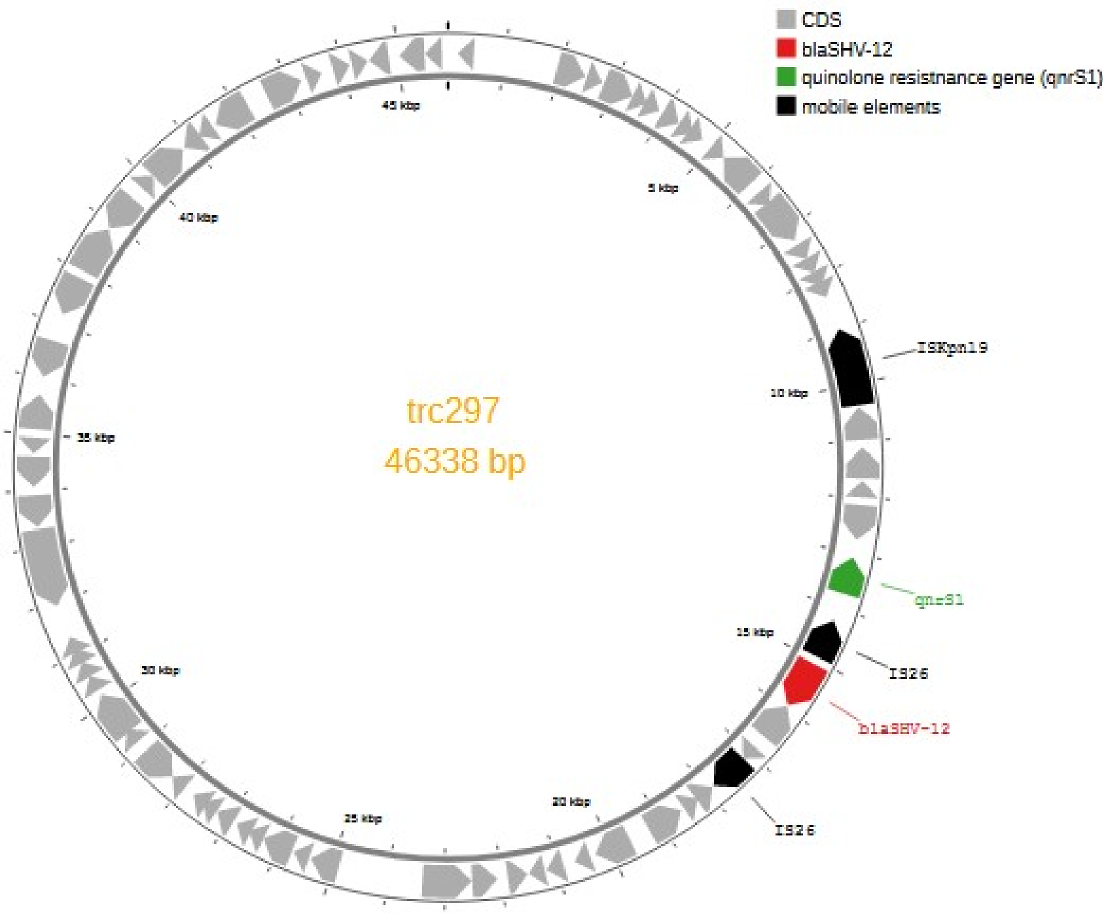
```
*****************************************************************************************************************************
Plasmid       Identity  Query / Template length     Contig                Position in contig    Note     Accession number
*****************************************************************************************************************************
IncFII             100  261 / 261                   11108795091_trc618_1  5025..5285            -        AY458016
=============================================================================================================================



Extended Output:

# IncFII_AY458016
template:       CACACCATCCTGCACTTACAATGCGCAGAAGGAGCGAGCACAGAAAGAAGTCTTGAACTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
query:          CACACCATCCTGCACTTACAATGCGCAGAAGGAGCGAGCACAGAAAGAAGTCTTGAACTT

template:       TTCCGGGCATATAACTATACTCCCCGCATAGCTGAATTGTTGGCTATACGGTTTAAGTGG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
query:          TTCCGGGCATATAACTATACTCCCCGCATAGCTGAATTGTTGGCTATACGGTTTAAGTGG

template:       GCCCCGGTAATCTTTTCGTACTCGCCAAAGTTGAAGAAGATTATCGGGGTTTTTGCTTTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
query:          GCCCCGGTAATCTTTTCGTACTCGCCAAAGTTGAAGAAGATTATCGGGGTTTTTGCTTTT

template:       CTGGCTCCTGTAAATCCACATCAGAACCAGTTCCTTGCCACCTTACGGCGTGGCCAGCCA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
query:          CTGGCTCCTGTAAATCCACATCAGAACCAGTTCCTTGCCACCTTACGGCGTGGCCAGCCA

template:       CAAAATTCCTTAAACGATCAG
                |||||||||||||||||||||
query:          CAAAATTCCTTAAACGATCAG
```

Figure 3.8 Results of PlasmidFinder regarding the plasmid compatibility groups a) ptrc203cli, b) ptrc297, and c) ptrc618

(a)


ptrc203cli
81582 bp
CDS
blaDHA-1 (β-lactamase, AmpC)
qnrB4 (fluoroquinolones resistance gene)
sul1 (sulfonamides resistance gene)
dfrA17 (trimethoprim resistance gene)
mobile elements
10 kbp
20 kbp
30 kbp
40 kbp
50 kbp
60 kbp
70 kbp
80 kbp
pspB
pspC
pspD
pspA
pspF
qnrB4
IS26
pemK
pemI
IS1D
Tn3
dfrA17
intI1
hybF
ampR
blaDHA-1
sul1
srpC
Transcriptional regulator
IS1R
stbB
parM
ybdJ
klcA
ltrA
ssb
noc
psiB
flmC
traM
traJ
traA
traV
traC
traN
traQ
ylpA
traD
traI
aidA
finO
yhcR
hha

(b)



CDS
blaSHV-12
quinolone resistnance gene (qnrS1)
mobile elements
trc297
46338 bp
5 kbp
10 kbp
15 kbp
20 kbp
25 kbp
30 kbp
35 kbp
40 kbp
45 kbp
ISKpn19
qnrS1
IS26
blaSHV-12
IS26

(c)

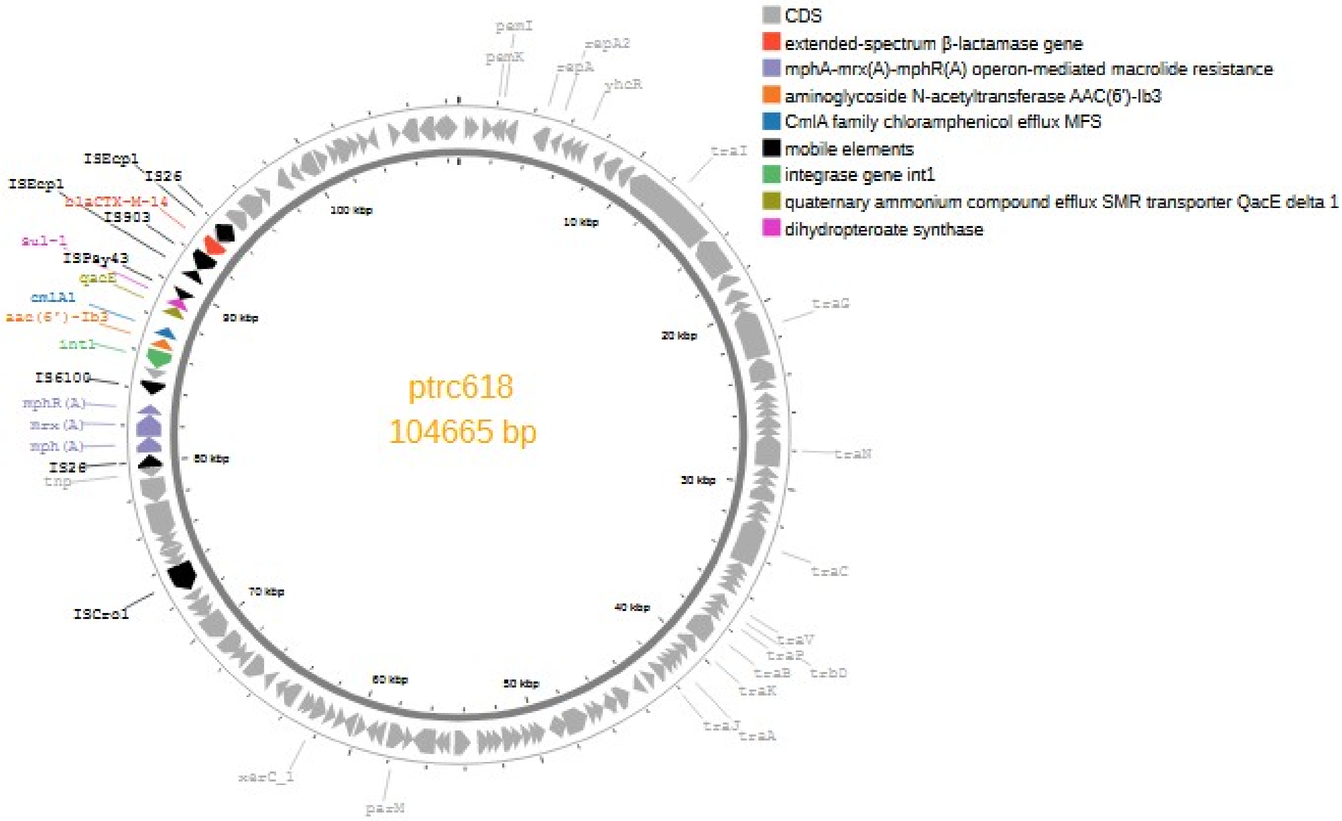
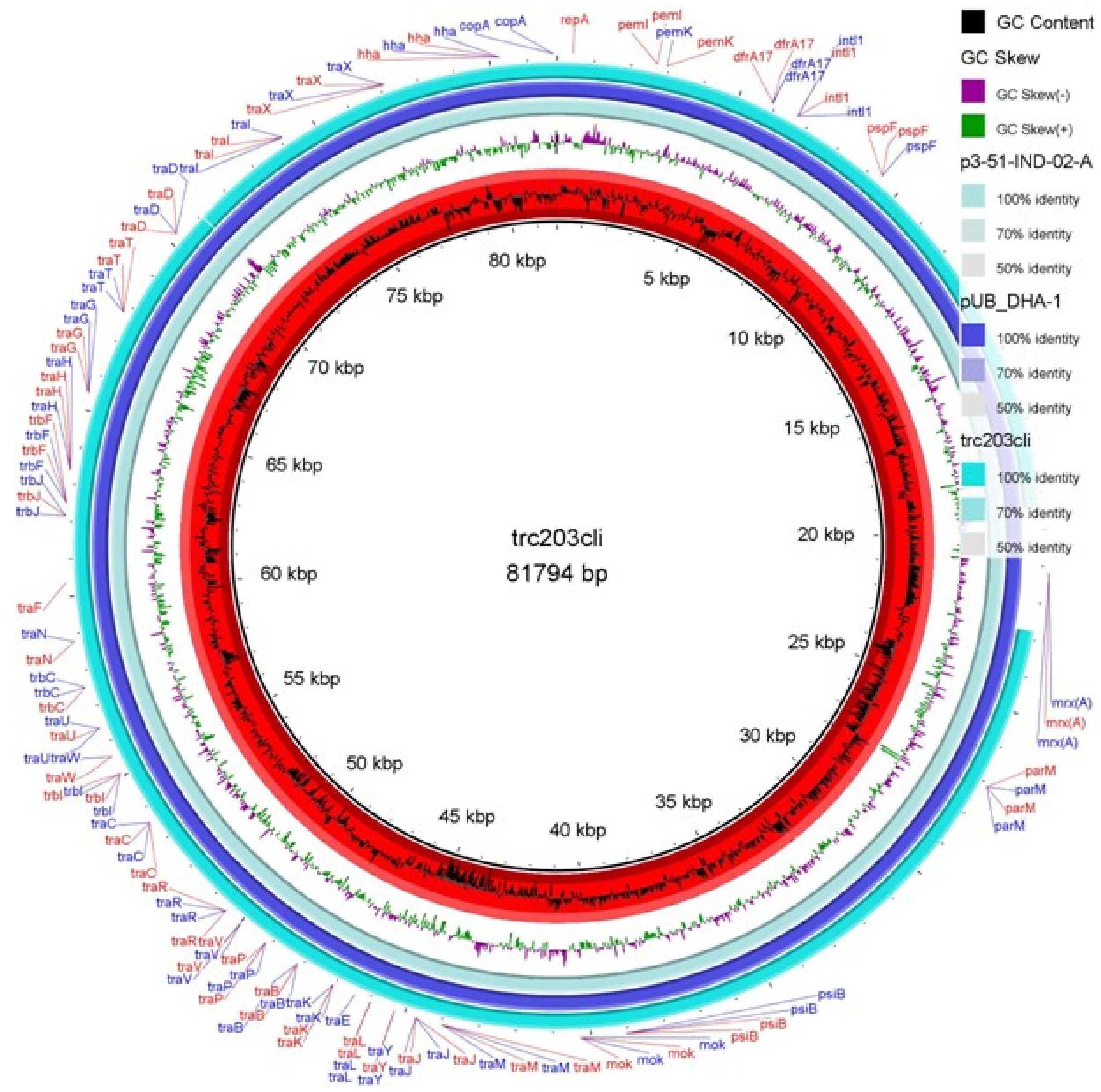
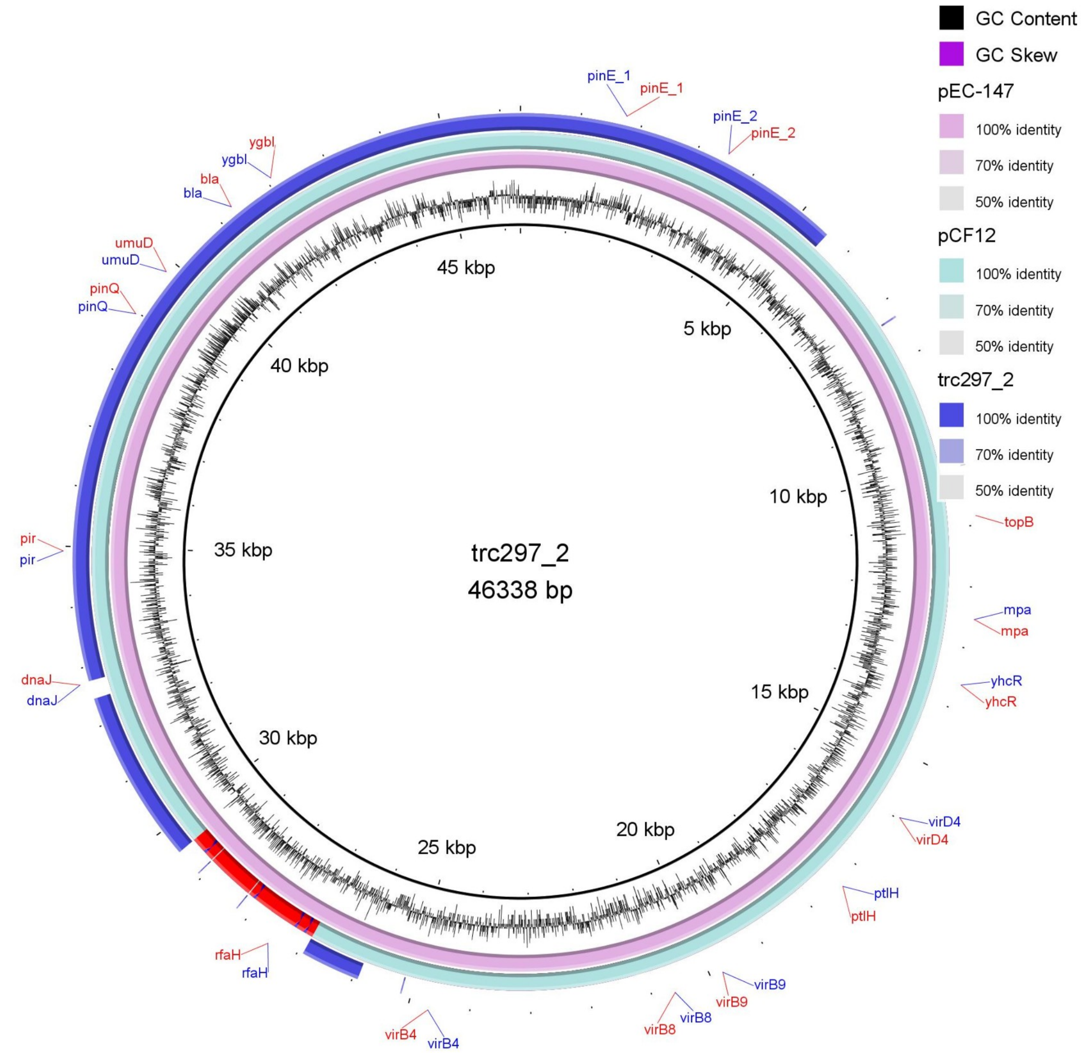


Figure 3.9 The plasmid maps constructed by proskee software and depict the resistance genes as well as the mobile elements which were identified in (a) ptrc203cli, (b) ptrc297 and (c) ptrc618.

(a)


trc203cli
81794 bp
5 kbp
10 kbp
15 kbp
20 kbp
25 kbp
30 kbp
35 kbp
40 kbp
45 kbp
50 kbp
55 kbp
60 kbp
65 kbp
70 kbp
75 kbp
80 kbp
GC Content
GC Skew
GC Skew(-)
GC Skew(+)
p3-51-IND-02-A
100% identity
70% identity
50% identity
pUB_DHA-1
100% identity
70% identity
50% identity
trc203cli
100% identity
70% identity
50% identity
repA
pemI
pemK
dfrA17
intI1
pspF
mrx(A)
parM
psiB
mok
traM
traJ
traY
traL
traE
traK
traB
traP
traV
traR
traC
trbI
traW
traU
trbC
traN
traF
trbJ
trbF
traH
traG
traT
traD
traI
traX
hha
copA

(b)


GC Content
GC Skew
pEC-147
100% identity
70% identity
50% identity
pCF12
100% identity
70% identity
50% identity
trc297_2
100% identity
70% identity
50% identity
trc297_2
46338 bp
5 kbp
10 kbp
15 kbp
20 kbp
25 kbp
30 kbp
35 kbp
40 kbp
45 kbp
pinE_1
pinE_1
pinE_2
pinE_2
topB
mpa
mpa
yhcR
yhcR
virD4
virD4
ptlH
ptlH
virB9
virB9
virB8
virB8
virB4
virB4
rfaH
rfaH
dnaJ
dnaJ
pir
pir
pinQ
pinQ
umuD
umuD
bla
bla
ygbl
ygbl

(c)

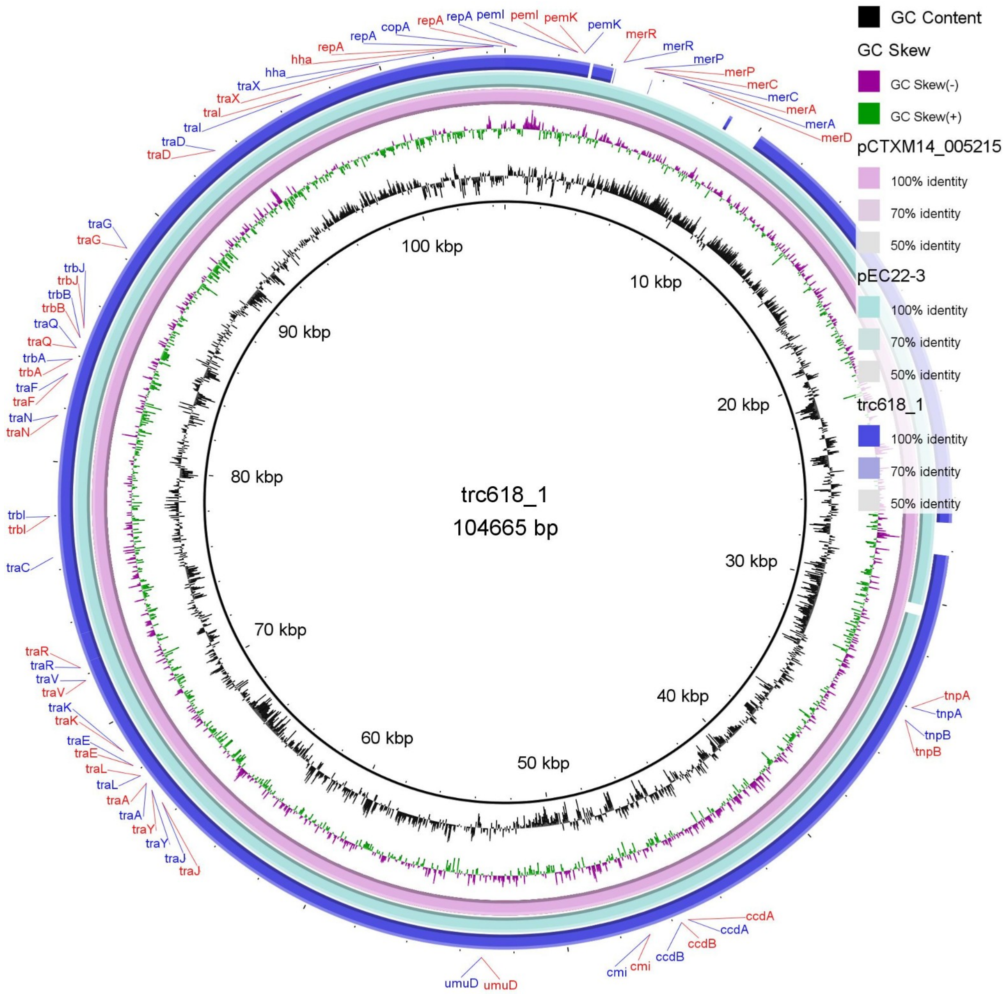


Figure 3.10 Comparative analysis of plasmids generated using BRIG. : (a) comparison between ptrc203cli, pUB_DHA-1 and p3-S1-IND-02-A, (b) comparison between ptrc297, pEC-147 and pCF12. (c) comparison between ptrc618, pCTX-M-14_005215 and pEC22-3.

4. Discussion

The rise of antibiotic resistant bacteria (ARB), particularly multidrug-resistant (MDR) Enterobacterales, is a growing global health crisis [**54, 55, 56**]. These resistant organisms pose a significant threat to public health due to the diminishing effectiveness of standard antibiotic treatments [**54, 55, 56, 57, 58**]. In this context, the present study focused on assessing antimicrobial resistance patterns, detecting antimicrobial resistance genes associated with resistant phenotypes, determining the genetic environment of resistance genes, and identifying molecular genotypes in *E. coli* isolates derived from various environments, including wastewater, river water, and clinical samples [**54, 56, 57, 58**]. The study's findings underscore the critical role of environmental reservoirs in the persistence and dissemination of antimicrobial resistance, reinforcing the need for a One Health approach to combat AMR.

The One Health approach, which emphasizes the interconnectedness of human, animal, and environmental health, has gained traction in the fight against AMR [**53, 54, 55**]. *E. coli* is a fundamental fecal indicator and a reliable marker for tracking AMR trends [**27, 40, 78**]. Monitoring *E. coli* in environmental samples provides crucial data for assessing the spread and dynamics of AMR. [**40, 78**].

Our study supports this notion by demonstrating that treated wastewater and surface waters harbor *E. coli* isolates resistant to commonly used antibiotics, such as ampicillin (AMP), ciprofloxacin (CIP), sulfamethoxazole/trimethoprim (SXT), and extended-spectrum cephalosporins (ESCs) [**8, 18, 19, 113**]. Our results indicate that resistance to penicillins (AMP and PIP) was the most prevalent among both environmental and clinical isolates, while a high quinolone (CIP) resistance rate was also observed in HWW (see **Figure 3.1**) [**114, 115, 116, 117**]. These findings are consistent with previous studies in Europe and North America, which have documented the persistence of ARB in treated wastewater and surface waters, often due to the incomplete removal of these organisms by conventional wastewater treatment plants (WWTPs). The detection of MDR *E. coli* in these environments is particularly concerning, as it suggests that these water bodies could act as reservoirs for ARB and ARG that may re-enter human populations through various pathways, including irrigation of crops, recreational water use, and contamination of drinking water supplies [**7, 23-26, 84-89**]. Moreover, the presence of *E. coli* as a fecal indicator organism in these settings highlights the potential for these environments to facilitate the transmission of pathogens, further exacerbating public health risks [**27, 40, 78**].

The study's findings also provide insights into the impact of the COVID-19 pandemic on AMR dynamics. The pandemic saw a significant increase in antibiotic use due to concerns about bacterial co-infections in COVID-19 patients, despite its viral nature [**118, 119, 120**]. Studies have reported that up to 70% of COVID-19 patients received antibiotics, even though bacterial co-infections were relatively rare, occurring in less than 10% of cases [**118, 119**]. This widespread use of antibiotics, particularly broad-spectrum agents like azithromycin and ceftriaxone, has raised concerns about the acceleration of AMR during the pandemic [**118- 121**]. Although our study did not find significant differences in resistance patterns between the pre-COVID and COVID periods,these results may be attributed to limitations such as restricted sampling opportunities during lockdowns.

The increased use of antibiotics during the pandemic could potentially have long-term consequences on the spread of AMR [**118-121**]. The over-prescription of antibiotics, especially when not clinically indicated, contributes to the selection pressure that drives the evolution and spread of resistant strains [**11-13**, **119**]. The potential for these resistant strains to enter environmental reservoirs, as suggested by our findings, further complicates efforts to control AMR, as these environments can serve as long-term reservoirs and sources of resistant bacteria.

Environmental settings, particularly water bodies contaminated by human and animal waste, have been identified as significant reservoirs for ARB, including several extended- spectrum cephalosporin- resistant *E. coli* (ESC-EC) [**27, 39, 40, 78-86, 113, 114**]. According to the results of this study, out of the 84 total ESC-EC isolates, the majority originated from wastewater (both hospital and treated wastewater) and involved ESBL-producing isolates. Specifically, ESBL-producers, particularly those from the CTX-M-group 1, which is the predominant type in both environmental and clinical settings, were widely isolated from various aquatic environments (such as rivers and lakes) as well as from hospitalized patients [**114 -117, 122, 123, 124**]. A portion of CTX-M-group1 producers isolated from patients' samples and wastewaters or river waters had the same resistance profiles, belonged to the same phylogenetic group and carried the same resistance gene (see **Table 3.6**). Isolates encoding blaCTX-M-group 9, another common subtype of blaCTX-M genes, were primarily found in river water samples, all of which shared the same plasmid pattern (see **Table 3.6**). Similarly to other studies [**24, 35, 34, 125, 126**], blaCTX-M-group 9, particularly the blaCTX-M-14 variant, are increasingly being detected in water sources, often linked to agricultural runoff and urban wastewater discharge. Furthermore, in hospital wastewater, blaSHV isolates were also commonly found, which may be related to the high prevalence of blaSHV-producing Enterobacteriales in clinical settings. [**35, 34, 125, 126**]. Regarding AmpC β-lactamase genes, two strains with blaCMY-4-like and one with blaFOX-17 were detected in hospital wastewater, while a clinical isolate with a blaDHA gene was also identified. In contrast to the widespread occurrence of ESC-EC, the presence of carbapenem-resistant *E. coli*, both in clinical and environmental populations, was very low. However, an isolate carrying the blaOXA-48 gene, and specifically the variant blaOXA-244, was isolated from river water. According to other studies, this variant has been detected in river water, estuaries, and even drinking water [**63, 78-90**].

According to the conjugation experiments conducted on a subset of ESC-EC strains, these genes were found to be located on conjugative plasmids, with a relatively high frequency of β-lactam resistance transfer. Moreover, in some conjugative clones, resistance to non-β-lactam antibiotics, such as nalidixic acid (NAL), ciprofloxacin (CIP), tobramycin (TOB), and sulfamethoxazole/trimethoprim (SXT), was also observed (see **Table 3.11**). This indicates that the acquired plasmid also co-transferred additional resistance genes, conferring resistance to other antibiotic groups such as (fluoro)quinolones and aminoglycosides, resulting in multidrug resistance. This observation was confirmed through the sequencing of three β-lactamase gene-carrying plasmids—ptrc203cli, ptrc297, and ptrc618—which harbored the genes blaDHA-1, blaSHV-12, and blaCTX-M-14, respectively (**Table 3.11**). Specifically, the plasmid ptrc203cli contained multiple antibiotic resistance genes (ARGs), including blaDHA-1 for resistance to extended-spectrum cephalosporins, sul1 for sulfonamides, dfrA17 for trimethoprim, and qnrB4 for reduced susceptibility to fluoroquinolones (**Table 3.12, Figure 3.9a**). Plasmid ptrc297 carried blaSHV-12, providing resistance to extended-spectrum cephalosporins, and qnrS1, which reduces susceptibility to quinolones (**Table 3.12, Figure 3.9b**). Plasmid ptrc618 harbored the mphA-mrx(A)-mphR(A) operon associated with macrolide resistance, along with aac(6')-Ib3 (aminoglycoside resistance), cmlA1 (chloramphenicol resistance), qacE (quaternary ammonium compounds resistance), and sul1 (sulfonamide resistance) (**Table 3.12, Figure 3.9c**). The plasmids ptrc203cli and ptrc618 were classified under the IncFII incompatibility group, while ptrc297 was categorized as IncX3. Both plasmid groups are conjugative and stable within bacterial hosts, commonly found in *E. coli, Klebsiella pneumoniae*, and other *Enterobacteriaceae* members, have been reported in both clinical and environmental isolates, and are associated with the dissemination of multidrug resistance [**127, 128]**. However, IncX3 plasmids are not as widespread as IncFII but have a somewhat broader host range compared to IncFII, potentially due to their smaller size and different replication mechanisms [**127, 128**]. Additionally, in all three of these plasmids, resistance genes or entire multidrug-resistant (MDR) regions were flanked by insertion sequence (IS) elements, particularly IS26, which facilitate the rearrangement and accumulation of resistance genes. This interplay between plasmids and IS elements significantly impacts the spread of multidrug resistance within bacterial populations [**27, 37, 38, 39, 41, 127, 128**].

To identify dominant genotypes in each habitat and their epidemiological relationships, the molecular technique of phylogrouping was applied. According to the phylogrouping results, in our clinical isolates, the B2 phylogroup predominated and was also the second most frequent group found in HWW and WWTP effluents (**Figure 3.4a, Table 3.9a**). Indeed, the B2 phylogenetic group has been previously reported to be dominant in hospital environments [**129-132**]. Isolates from both groups B2 and D, known for their pathogenic potential, possessed the chuA gene[**108**], responsible for hemin utilization [**129- 134]**. This indicates a strong correlation between pathogenicity and phylogenetic groups B2 and D. In our study, a portion of clinical and environmental MDR and resistant (R) isolates were classified into phylogenetic groups B2 and D (**Figure 3.4b**), highlighting the significant human health risks associated with potential pathogenic R and MDR *E. coli* isolates from environmental sources such as rivers.

To detect potential changes in the frequency of each phylogenetic group in different environments before and during the COVID-19 pandemic, we observed that in the winter of 2019-2020 and the spring of 2021 (the second wave of COVID-19), the B2 group had the highest frequencies observed among environmental isolates, at 35% and 28%, respectively (**Figure 3.5**). Additionally, among environmental isolates, an increase in group D was noted in the autumn of 2020 (the first wave of COVID-19), with a frequency of 23% (**Figure 3.5**). Regarding clinical strains, a significant rise in group B2 was observed in the autumn of 2019 and the summer of 2020 (the first wave of COVID-19), while group D increased in the autumn of 2020 (the first wave of COVID-19) and the spring of 2021 (the second wave of COVID-19) (**Figure 3.5**), suggesting that pandemic-related changes in environmental conditions and human activity influenced microbial population dynamics..

Although Pulsed-Field Gel Electrophoresis (PFGE) is considered the gold standard for epidemiological analysis and a valuable tool for detecting nosocomial outbreaks, it is less effective when dealing with non-clonal, genetically diverse populations found in broader environments such as wastewater or river water. This makes it inadequate for identifying subtle molecular differences. This limitation has been demonstrated in several studies [**135, 136, 137**], as well as in our own findings, where PFGE revealed considerable heterogeneity, even among strains from clinical settings and hospital wastewater (HWW), which share a close epidemiological relationship. Furthermore, significant heterogeneity was observed even among the clinical isolates, which is expected, as most of these strains were not related to nosocomial infections. Therefore, this method did not provide additional insights into the molecular classification of the *E. coli* isolates.

To sum up, the reported results reveal that treated wastewater and river water are sources of resistant bacteria. The potential reuse of treated wastewater and river water, particularly for restricted crop irrigation depending on the method of watering (e.g., spraying), may expose humans to the risk of developing gastroenteritis, especially via droplet ingestion [**138, 139, 140**]. As E. coli is the leading cause of both community- and hospital-acquired UTIs, the detection of MDR strains in environmental samples raises significant public health concerns. For UTI treatment, the recommended antimicrobials are SXT, CIP, and AMC. In our study, *E. coli* strains that were found to be multidrug-resistant (MDR), including those with co-resistance to SXT, CIP, and AMC (**Table**

3.4), were isolated not only from the biological fluids of patients but also from all environmental habitats (see **Figure 3.1**). This demonstrates that human health risks can arise from exposure to MDR *E. coli* isolates present in waste and aquatic environments.

In our study, due to strict lockdown measures imposed during the COVID-19 pandemic, we were unable to carry out some samplings, which made seasonal analysis infeasible. Additionally, the molecular typing techniques employed did not provide adequate clustering information regarding the circulation of specific *E. coli* types between clinical settings and the environment. Nevertheless, this study represents the first systematic collection of *E. coli* isolates obtained from wastewater and river water samples from Livadeia, Greece, an area that combines urban life, husbandry, and agriculture. Despite these limitations, this study provides valuable insights into *E. coli* resistance profiles, the genotypes present, and the resistance mechanisms involved in the spread of resistance in wastewater and aquatic habitats. Future efforts will focus on conducting plasmid sequencing on additional *E. coli* isolates. This approach will yield comprehensive insights into the genetic context of circulating resistance genes and elucidate the molecular mechanisms contributing to antimicrobial resistance in these bacteria. The presence of antimicrobial-resistant (*AR*) *E. coli* isolates with the same multidrug-resistant profiles (MRPs) in clinical and hospital wastewater (HWW) samples sheds light on the spread of resistant bacteria in water bodies. The reported findings suggest a potential exchange of *AR* bacterial populations and similar *AR* determinants between clinical and environmental habitats. This raises concerns for public health, as aquatic environments could serve as reservoirs for the transmission of resistance genes to various bacterial species.

References

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