CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA

PORTO

CHARACTERIZATION OF ENVIRONMENTAL AND CLINICAL ANTIBIOTIC-RESISTANT *Escherichia Coli* ISOLATES

by

Daniel Fernandes Magalhães Filipe

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

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Resumo

A rápida disseminação de resistência a antibióticos entre bactérias patogénicas humanas tem sido uma problemática de saúde pública global, em expansão nas últimas décadas. Os genes de resistência a antibióticos encontrados em bactérias patogénicas clínicas não são o único motivo de preocupação. Também as bactérias comensais e ambientais podem transportar genes de resistência, por vezes disseminados através de processos de transferência horizontal de genes, mediados por elementos genéticos móveis, em combinação com seleção.

O processo de conjugação é o mecanismo de transferência horizontal de genes que se considera ser o mais relevante na disseminação da resistência a antibióticos. Pensa-se que a disseminação de genes de resistência possa ser intensificada na presença de diversas substâncias como sais de metais ou antibióticos. Conhecer as condições em que a mobilização pode ser estimulada é crucial para poder controlar a disseminação.

Este estudo teve como objetivo comparar isolados de *Escherichia coli* de amostras clínicas e de águas residual e de superfície (n = 52), de modo a avaliar se os perfis de resistência eram dependentes da origem. Um segundo objetivo foi o de compreender se a taxa e perfil de transferência de genes de resistência a antibióticos diferia consoante a temperatura de conjugação. O trabalho envolveu caracterização fenotípica, genotípica e pesquisa de genes de resistência, bem como ensaios de conjugação entre uma estirpe resistente a carbapenemos e uma receptora de referência. Especificamente, foi determinada a suscetibilidade a diferentes classes de antibióticos pelo método de difusão em disco, foi realizada a deteção por PCR de genes de resistência a antibióticos e de tipos de replicões presentes em plasmídeos. Nos ensaios de conjugação, testaram-se diferentes temperaturas (25, 28, 35 e 40ºC), os transconjugados foram selecionados na presença de azida e ceftazidima, analisados por genotipagem para verificar a sua autenticidade e caracterizados para a presença de determinantes genéticos específicos.

A caracterização genotípica e fenotípica de isolados presumivelmente identificados como *E. coli* não revelou diferenças significativas ($p>0.05$) entre isolados ambientais e clínicos, com a exceção da prevalência de plasmídeos de replicão do tipo IncF, significativamente ($p<0.05$) mais elevada em isolados clínicos. Apenas um isolado de *E. coli* apresentou o gene bla_{KPC} e demonstrou fenótipo de resistência ao antibiótico meropenemo. As taxas de conjugação não se revelaram significativamente diferentes ($p>0.05$) a diferentes temperaturas. A transmissão do plasmídeo de replicão tipo IncN e que continha o gene bla_{KPC} foi observada em todos os transconjugantes analisados. No entanto, a transmissão do replicão do tipo FIB foi mais elevada a temperaturas de conjugação de 35 e 40ºC do que a 25 e 28ºC.

Palavras-chave

Escherichia coli, resistência a antibióticos, carbapenemos, temperatura, taxa de conjugação

Abstract

The rapid spread of antibiotic resistance among human pathogenic bacteria has been a global public health problem, expanding in recent decades. Antibiotic resistance genes found in clinical pathogens are not the only cause for concern, as it is recognized that commensal and environmental bacteria, as well as their mobile genetic elements, can function as reservoirs and vectors of resistance, and can spread genes through horizontal transfer and selection.

The conjugation process is the horizontal gene transfer mechanism considered to be the most relevant in resistance spread. It is thought that the spread of resistance genes can be enhanced in the presence of various substances such as metal salts or antibiotics. Knowing the conditions under which mobilization takes place is crucial to control resistance spread.

This study aimed to compare *Escherichia coli* isolates from clinical, wastewater and surface water samples (n=52), in order to assess if resistance profiles were source-dependent. A second objective was to understand if the rate and profile of antibiotic resistance gene transfer differed depending on the conjugation temperature. This work involved phenotypic and genotypic characterization and detection of resistance genes, as well as conjugation assays between a carbapenem resistant strain and a reference receptor. Specifically, susceptibility to different classes of antibiotics was determined by the disk diffusion method, PCR detection of antibiotic resistance genes and types of replicons present in plasmids was performed. In the conjugation assays, different temperatures were tested (25, 28, 35 and 40ºC), the transconjugants were selected in the presence of azide and ceftazidime, confirmed by genotyping and characterized for the presence of specific genetic determinants.

Genotypic and phenotypic characterization of isolates identified as *E. coli* did not reveal significant differences ($p>0.05$) between environmental and clinical isolates, with the exception of IncF-type replicon plasmids, which were significantly ($p<0.05$) more prevalent among clinical isolates. Only one *E. coli* isolate was observed to harbour the bla_{KPC} gene and demonstrated a meropenem-resistance phenotype. Conjugation assays revealed no significant differences ($p>0.05$) between the rates observed at different temperatures. The transmission of the IncN plasmid holding the bla_{KPC} gene was detected in all transconjugants analysed. However, the transmission of the FIB-type replicon was higher at conjugation temperatures of 35 and 40ºC than at 25 and 28ºC.

Keywords

Escherichia coli, antibiotic resistance, carbapenems, temperature, conjugation rate

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Contents

Resumo III

Abstract V

Acknowledgments VII

Figure list XI

Table List XIII

Abbreviations XV

1 Introduction 17

1.1 *Escherichia coli* and other *Enterobacteriaceae* 17

1.2 Genetic analysis aiming at bacterial identification 17

1.2.1 PCR amplicon sequencing 18

1.3 Classes of antibiotics 19

1.3.1 Carbapenems 21

1.4 Antibiotic resistance 22

1.4.1 β-lactamase encoding genes 23

1.5 Importance of environment for antibiotic resistance dissemination 26

1.6 Horizontal gene transfer mechanisms 27

1.7 Mobile genetic elements: Plasmids and incompatibility groups; Integrons 29

2 Objectives 30

3 Methods 31

3.1 Bacterial strains 31

3.1.1 Bacteria and characterization 31

3.1.2 Antibiotic Susceptibility testing 31

3.1.3 Genotyping and gene detection 31

3.2 Conjugation assays 33

3.3 Statistical analysis 34

4 Results / Discussion 35

4.1 Isolates identification 35

4.2 Phenotypic characterization 36

4.3 Genotypic characterization 37

4.4 Effect of temperature on conjugation rate and transconjugant profile 40

5 Conclusion 44

6 Future work 45

References 45

Figure list

Figure 1: Antibiotics target sites. 21
Figure 2: General carbapenem core structure, and most significant antibiotics of this class. 22
Figure 3: Mechanisms and targets of antibiotic resistance. 23
Figure 4: Antibiotic resistance spread between environmental and clinical settings. 26
Figure 5: Mechanisms of horizontal gene transfer. 27
Figure 6: Percentage values of species identified based on 16S rRNA gene sequence analysis 35
Figure 7 Percentage values of resistant clinical and environmental isolates for each tested antibiotics 36
Figure 8: Percentage of multidrug-resistant isolates. 37
Figure 9: Prevalence values of selected resistant genes among tested Isolates from clinical and environmental settings 38
Figure 10: Validated transconjugants counts mean, viable donor counts mean, and validated conjugation rate at each temperature. 42
Figure 11: Percentage of replicon type FIB positive transconjugants harboring bla_{SHV} and *intI1* genes.. 43

Table List

Table 1: Different antibiotic classes and respective characteristics 20
Table 2: β-lactamases Ambler classification 24
Table 3: Example of mobile genetic elements involved in resistance spread and respective characteristics 29
Table 4: Primers and conditions used in PCR analysis of different selected genes 32
Table 5: Antibiotic resistance phenotypes and genotypes of the Enterobacteriaceae isolates with clinic and environmental origin 39
Table 6: Number of viable donors, transconjugants replicate means, percentage of RAPD validated transconjugants, conjugation rate and respective standard deviation 41

Abbreviations

ARG Antibiotic resistance genes

ARB Antibiotic resistant bacteria

CAZ Ceftazidime

CCA Chromogenic Coliform Agar

CIP Ciprofloxacin

CN Gentamicin

CTX Cefotaxime

ESBL Extended-spectrum β-lactamase

MEM Meropenem

PCA Plate count agar

PCR Polymerase chain reaction

TE Tetracycline

RAPD Random amplified polymorphic DNA

SXT Trimethoprim-sulfamethoxazole

1 Introduction

1.1 *Escherichia coli* and other *Enterobacteriaceae*

Recently, the World Health Organization (WHO) established a critical group of bacteria posing a major threat to human health. Included in this list are different members of the family *Enterobacteriaceae*, such as the species *Escherichia coli* and *Klebsiella pneumoniae*. Both organisms are classic opportunistic pathogens that may be acquiring new genes to resist antibiotics and continue to be successful colonizers (Tacconelli et al., 2018).

The Gram-negative *Escherichia coli* is not confined to clinical settings, being a ubiquitous constituent of the environmental bacterial microbiota and in the feces of birds and mammals. This organism distribution has been universally used as an indicator of fecal contamination and water quality (Ferreira et al., 2019; Higgins et al., 2007; Rocha et al., 2022).

E. coli is one of the most interesting bacterial groups for source tracking studies and to infer about the processes and paths of resistance dissemination. The fact that this species is known to harbor plasmids capable of acquiring different families of antibiotic resistance genes, combined with the emergence of multidrug-resistant *E. coli* strains, emphasizes the significance of surveying antibiotic resistance determinants in environmental populations of these bacteria (Varela et al., 2015).

1.2 Genetic analysis aiming at bacterial identification

The 16S ribosomal RNA (16S rRNA) is the RNA component of the small subunit (30S) of prokaryotic ribosomes. The analysis of the sequence of gene that encodes the 16s rRNA is a powerful tool for deducing phylogenetic and evolutionary relationships, operating as a reliable “molecular clock”, as it is highly conserved between different distantly related species of bacteria and archaea (Weisburg et al., 1991; Yang et al., 2016). Full-length 16S rRNA gene sequences consist of nine hypervariable regions separated by nine highly conserved regions (Johnson et al., 2019; Yang et al., 2016). While in the characterization of microbial communities are examined short reads (~300 bp) of this gene, for the identification of bacterial isolates is possible to analyze the nearly full-sequence (<1500 bp) (Johnson et al., 2019). As a basic criterion it is assumed that sequences sharing > 97% identity may belong to the same species (Johnson et al., 2019).

In contrast with the universal distribution of the ribosomal rRNA gene, some genes can be used as biomarkers of a given species. An example of this is the *lacZ* gene, related with lactose metabolism, encoding the enzyme β-galactosidase, responsible for the cleavage of lactose into glucose and galactose (Li et al., 2007). This gene is present in *E. coli* but not in most of the related Gram-negative oxidase-negative rods, permitting a fast distinction of taxa. A 365-bp fragment of *lacZ* gene has been used to screen *E. coli* among isolates with similar features, isolates of *K.*

oxytoca, *Shigella* spp., and *Enterobacter* spp. may be responsible for some false positive results (Higgins et al., 2007).

1.2.1 PCR amplicon sequencing

The detection of a gene based on PCR amplification may be a result in itself, however, the analysis of the respective DNA sequence is always necessary. It may be needed to confirm the authenticity of the PCR amplicon (this is the case of the gene lacZ) or it may be crucial to determine sequence identity values (this is the case of the 16S rRNA gene). Although next generation sequencing methods are available, the first generation methods - Sanger sequencing, also known as the “chain termination method”, is the method of choice for these purposes. This method was firstly developed by Nobel Prize winner Frederick Sanger in 1977. It can be performed manually, although it is normally carried out using automated sequencing machines. In both situations, the process requires three main steps comprising chain-termination PCR, size separation by gel electrophoresis, and sequence determination (Wong et al., 2019).

Chain-termination PCR operates with the inclusion of a low ratio of chain-terminating modified nucleotides denominated dideoxyribonucleotides (ddNTPs). Opposite to common nucleotides, these lack the 3'-OH group required for phosphodiester bond formation between one nucleotide and the next, in the standard DNA elongation. Consequently, when DNA polymerase incorporates a ddNTP at random, it inhibits further strand extension, resulting in millions to billions of oligonucleotide copies of the DNA sequence of interest, terminated at diverse lengths (Deng et al., 2015). Manual Sanger sequence uses four PCR reactions, each with only a single type of ddNTP (ddATP, ddTTP, ddGTP, and ddCTP). In automated sequencing, all ddNTPs are mixed, each having a unique fluorescent label, requiring a single reaction. These fragments are then separated by size using gel or capillary tube electrophoresis and the identity of each terminal ddNTP is used to determine the final sequence (Deng et al., 2015; Senabouth et al., 2020).

Numerous databases and data analysis platforms exist for analysing DNA sequencing data. Most of these have specific purposes, such as determine the taxonomic relationships among *Bacteria* or *Archaea*, based on 16S rRNA gene sequence for all terminal taxa (species or subspecies). An example of this is the EzBioCloud platform (www.ezbiocloud.net) that provides the reliable identification of bacteria based on the 16S rRNA gene (Yoon et al., 2017).

1.3 Classes of antibiotics

Antibiotics are organic compounds which at low concentrations can inhibit the growth (bacteriostatic) or kill (bactericidal) bacteria. Antibiotics can be of biological origin (produced by microorganisms), or, more recently, produced partly or entirely through synthetic means (W.B. Hugo & A.D. Russell, 2004).

Penicillin was the first antibiotic discovered by Sir Alexander Fleming in September 1928, obtained from a soil fungus. The development and further introduction of this antibiotic into the health care system in the 1940s has transformed the management of bacterial infections (Aminov, 2010).

The most standard antibiotic classification schemes are based on their molecular structures, method of action, and spectrum of activity (Table 1). Common classes include β-lactams, macrolides, tetracyclines, quinolones, aminoglycosides, sulphonamides, glycopeptides, and oxazolidinones (Etebu & Arikekpar, 2016; Shifa Begum et al., 2021).

Table 1: Different antibiotic classes and respective characteristics. Source: (Bush et al., 2020; Etebu & Arikekpar, 2016; Nurjadi et al., 2021; Shifa Begum et al., 2021; Tačić et al., 2017; W.B. Hugo & A.D. Russell, 2004; Zhong et al., 2022)

Antibiotics	Structure	Mode of action	Activity spectrum	Examples
β-lactam	Highly reactive 3-carbon and 1-nitrogen ring	Interferes with the synthesis of the bacterial cell wall, resulting in lysis and cell death	Effectiveness against Gram-negative pathogens	Penicillins, cephalosporins, monobactams, and carbapenems
Sulfonamides	Sulfonamide group and an amino group in the *para* position of the benzene ring	Competitive inhibition of folic acid synthesis and consequent cell reproduction and growth blockage	Broad-spectrum activity, effective against Gram-positive and certain Gram-negative bacteria	sulfamethoxazole
Aminoglycosides	Amino sugars attached to an aminocyclitol ring by glycosidic bonds	Target bacterial ribosomes, inducing mRNA misreading or inhibiting translocation	Aerobic Gram-negative bacteria and some staphylococci strains	streptomycin, neomycin, gentamicin, kanamycin
Tetracyclines	Four cyclic rings	Target the cell's small ribosomal subunit and protein synthesis	Broad-spectrum antibiotics, active against Gram-positive and Gram-negative bacteria	tetracycline, chlortetracycline, methacycline, tigecycline
Quinolones	Two rings, with the possible addition of more rings through recent generations	Target the bacterial enzymes DNA gyrase and DNA topoisomerase IV, inhibiting bacterial DNA replication and transcription	Several different types of Gram-negative bacteria; fluoroquinolone generations exhibit enhanced activity against *Enterobacteriaceae* and *Pseudomonas aeruginosa*	nalidixic acid, ciprofloxacin, norfloxacin

Antibiotics' action is directed at some specific feature of the bacterial structure or their metabolic processes. The most common mechanisms of antibiotic actions are the inhibition of cell wall synthesis, inhibition of the structure and function of nucleic acids, inhibition of protein synthesis,

or blockage of key metabolic pathways (Etebu & Arikekpar, 2016). Several different antibiotics and their active sites are presented in figure 1.

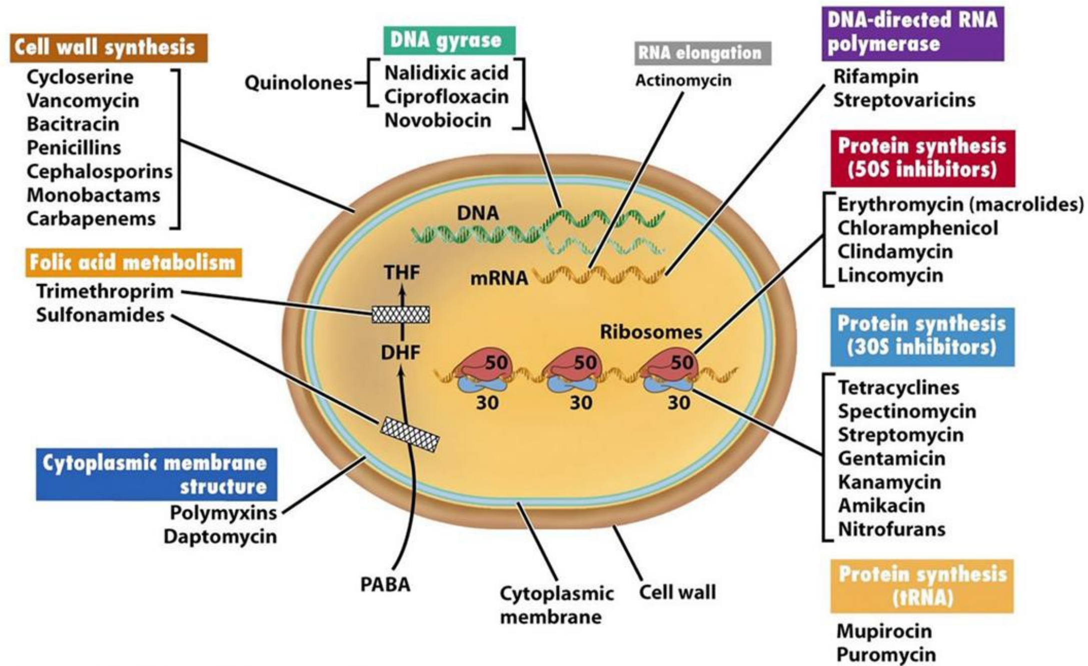


Figure 1: Antibiotics target sites. Source: (Etebu & Arikekpar, 2016)

1.3.1 Carbapenems

Carbapenem antibiotics are considered last defense drugs, against which resistance has been increasing. These antibiotics were discovered in 1976 to fight the great emergence and activity of β-lactamase enzymes. These antibiotics are extremely important and represent a central role in the fight against bacterial infections, as they resist and inhibit the hydrolytic action of β-lactamase enzymes and present the broadest spectrum of activity among β-lactam antibiotics. For these reasons, this class is considered to include last-resort antibiotics prescribed to treat drug-resistant bacterial infections and severe state diseases (Shifa Begum et al., 2021; Sotello et al., 2018). The most significant examples commonly administered are imipenem, meropenem, and ertapenem, all broad-spectrum antibiotics (figure 2). Imipenem is effective, at very low concentrations, against aerobic and anaerobic pathogens. Meropenem is effective against non-fermentative Gram-negative bacilli. Ertapenem, on the other hand, shows limited activity against non-fermentative Gram-negative bacilli (Etebu & Arikekpar, 2016; W.B. Hugo & A.D. Russell, 2004). However, the rapid emergence of bacterial pathogens resistant to carbapenems, for example, by carbapenemase production, has become a global health concern. Carbapenem resistant infections have been reported to have an associated mortality rate between 40–80% (Sotello et al., 2018).

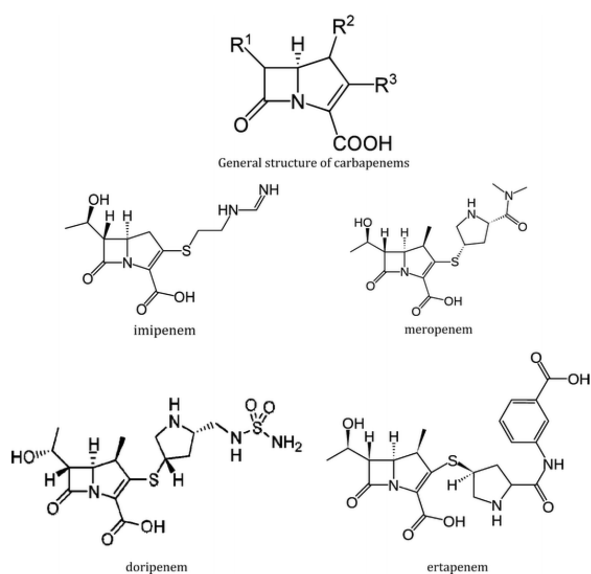


Figure 2: General carbapenem core structure, and most significant antibiotics of this class. Source: (Finberg & Guharoy, 2012)

1.4 Antibiotic resistance

Since the 1970s, the discovery of new novel antibiotic classes abruptly dropped. The scarcity of development of new antibiotics and the rapid emergence of resistant strains even to last-resort antibiotics, emphasizes the importance of conserving available antimicrobials (Aminov, 2010; Manaia, 2017; von Wintersdorff et al., 2016). Thus, the emergence of multi-drug resistant bacteria to several classes of antibiotic agents, represents today's major clinical problem (W.B. Hugo & A.D. Russell, 2004).

The alarming spread of resistance by horizontal gene transfer is largely due to selective pressure through human antibiotic use and misuse (von Wintersdorff et al., 2016). Bacteria surviving in environments presenting antibiotics were able to naturally evolve mechanisms to overcome their antimicrobial activities. Most fundamental mechanisms involve enzymatic degradation of antibiotics, alteration of bacterial proteins that are antimicrobial targets, and change the membrane permeability to antibiotics (Larsson et al., 2018; Manaia, 2017; Rocha et al., 2022; Shifa Begum et al., 2021; Vaz-Moreira et al., 2011). The main mechanisms of antibiotic resistance are illustrated in figure 3.

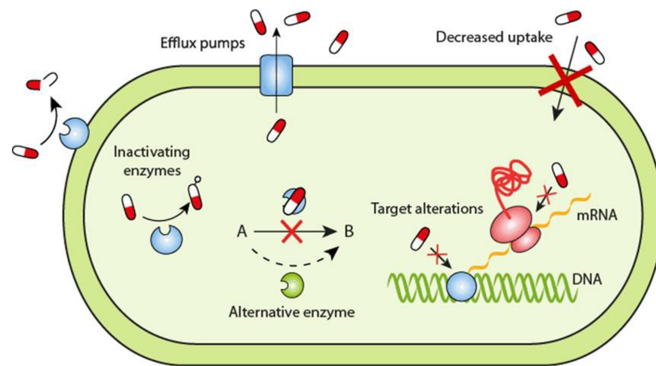


Figure 3: Mechanisms and targets of antibiotic resistance. Source: (Shifa Begum et al., 2021)

1.4.1 β-lactamase encoding genes

Resistance to β-lactam antibiotics is an increasing problem and β-lactamase production is the most common mechanism of drug resistance, especially in Gram-negative bacilli (Dallenne et al., 2010; Henriques et al., 2006; Manaia et al., 2018; Paterson et al., 2003; Rocha et al., 2022; Singh et al., 2020). β-Lactamases are enzymes that share the characteristic of catalyzing the ring-opening of the β-lactam antibiotics. Ambler classification system, based on peptide sequence, include four classes from A to D (Table 2). These enzymes are generally plasmid-mediated and can be responsible for outbreaks through horizontal gene transfer (Dipersio et al., 2005; Paterson et al., 2003).

Table 2: β-lactamases Ambler classification. Source: (Carattoli et al., 2005; Dallenne et al., 2010; Dipersio et al., 2005; Gootz et al., 2009; Henriques et al., 2006; Manaia et al., 2018; Paterson et al., 2003; Pfeifer et al., 2010; Rocha et al., 2022; Singh et al., 2020; W.B. Hugo & A.D. Russell, 2004)

β-Lactamase classes	**Structure**	**Type**	**Spectrum**	**Bacterial host**	**Examples**
A	Serine at the active site	Broad-spectrum β-lactamases	Hydrolyze ampicillin and penicillin	*Enterobacteriaceae* and non-fermenters	TEM-1, TEM-2, SHV-1,
		Extended-spectrum β-lactamases	Penicillins, 3rd generation cephalosporins		SHV-2, CTX-M, PER, VEB
		Carbapenemases	Hydrolyze carbapenems and all other β-lactams		KPC, IMI, SME
B	Four zinc atoms at their active site	Metallo-β-lactamases	Hydrolyze carbapenems		VIM, IMP, NDM
C	Serine at the active site	Cephalosporinases chromosomally encoded	Hydrolyze cephamycins and some oxyimino β-lamtams; inducible, but mutation can lead to overexpression	*Enterobacter* spp. *Citrobacter* spp.	AmpC
		Cephalosporinases plasmid encoded			CMY, DHA
D		OXA-type enzymes	Hydrolyze oxacillin, oxyimino β-lamtams, and carbapenems;	*Enterobacteriaceae*; *Pseudomonas aeruginosa* and *Acinetobacter baumannii*	OXA

Gram-negative bacteria attempt multiple molecular approaches for the development of resistance to β-lactam antibiotics. Some of these strategies comprise the creation of wider extended-spectrum β-lactamases (ESBL) by mutation of previous extensively disseminated plasmid-encoded β-lactamases by one or more amino acid substitution; acquisition of genes encoding ESBL from environmental bacteria; high-level expression of chromosome-encoded β-lactamase genes; or mobilization of plasmid-mediated *bla* genes by horizontal transfer into other species (Dipersio et al., 2005; Larsson et al., 2018; Pfeifer et al., 2010; Bertrand et al., 2006; Dallenne et al., 2010). ESBLs (extended-spectrum β-lactamases) are generally derived from *TEM-1*, *TEM-2*, and *SHV-1* β-lactamases by base pair mutations or are from a rapidly evolving class called *CTX-M (originally acquired* from environmental bacteria) (Dipersio et al., 2005). Other Ambler class A β-lactamases can confer resistance to carbapenems, such as *KPC* carbapenemases (Gootz et al., 2009).

1.5 Importance of environment for antibiotic resistance dissemination

Impacts of the contaminant resistome (linked to human activities) on environmental antibiotic-resistant reservoirs are recognized mainly in areas under human anthropogenic influence. Overlap of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), between the human microbiome and potential environmental sources, have been increasingly recognized as a health risk (Larsson et al., 2018; Manaia, 2017; Manaia et al., 2018; Rocha et al., 2022; Vaz-Moreira et al., 2011). Figure 4 illustrates the pathways for antibiotic resistance spread between clinical and environmental settings.

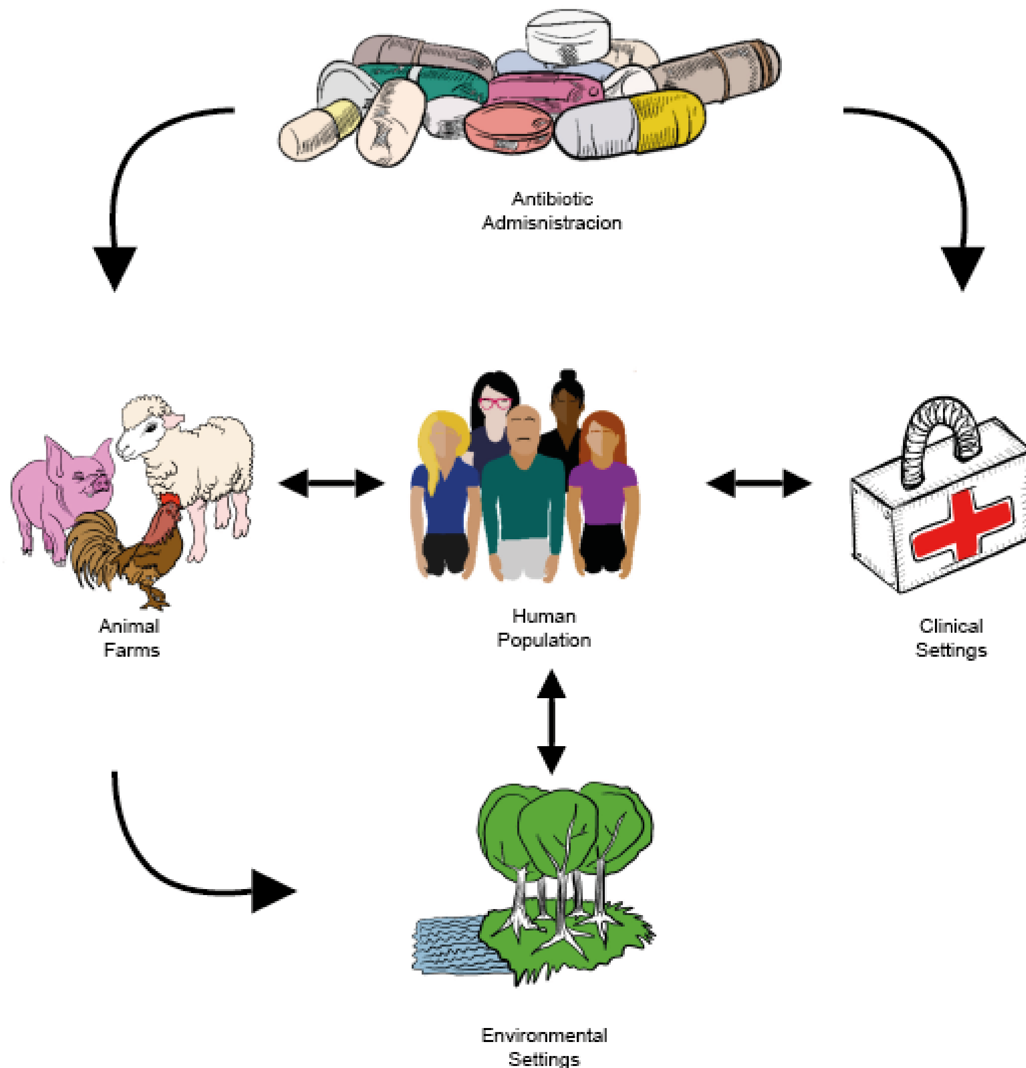


Figure 4: Antibiotic resistance spread between environmental and clinical settings. Adapted from: (Larsson & Flach, 2021)

1.6 Horizontal gene transfer mechanisms

Gene transfer can be vertical or horizontal. Vertical gene transfer occurs when the genetic material is transmitted from parent to offspring, whether by sexual or asexual reproduction, whereas horizontal gene transfer happens between two existing organisms, where a donor organism transfers genetic material to a recipient organism (Sun et al., 2019; von Wintersdorff et al., 2016). Transformation, transduction and conjugation comprise the main mechanisms of horizontal gene transfer (figure 5). Horizontal Gene transfer, in particular the conjugation pathway, is unanimously considered in the literature as the main responsible in today's antibiotic resistance epidemic (von Wintersdorff et al., 2016).

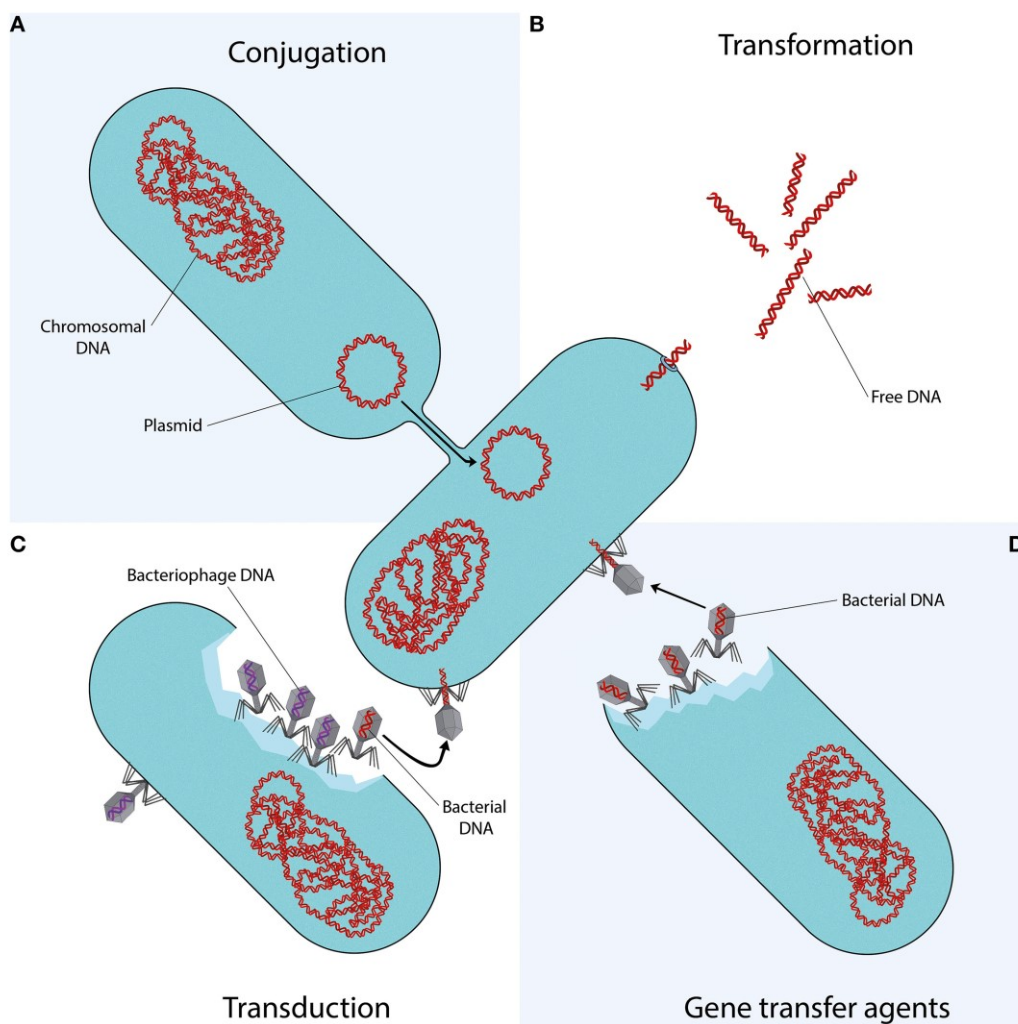


Figure 5: Mechanisms of horizontal gene transfer. Source: (von Wintersdorff et al., 2016)

The transformation process consists on the uptake, integration, and functional expression of extracellular free DNA fragments, by certain bacteria. This process requires several conditions such as the presence of extracellular DNA, bacterial ability to receive this free DNA (state of competence), and DNA integration into the recipient genome. Exposure to antibiotics can induce competence in many species of bacteria, which further stimulates resistant genes transformation (von Wintersdorff et al., 2016). Bacteriophages play a meaningful role in shaping the bacterial microbiome in any environment. Through transduction, bacteriophages can transfer genes that are advantageous to their microbial hosts in order to replicate themselves. Studies have found bacteriophages carrying β-lactamase genes in *E. coli*.(von Wintersdorff et al., 2016) Treatment with antibiotics increases the number of ARGs in phages and expands the interactions between phage and bacterial species (von Wintersdorff et al., 2016). Conjugation is a process of transfer of genetic material through a method requiring cell-to-cell contact through cell surface pili (von Wintersdorff et al., 2016). This process is facilitated by the conjugative machinery which is encoded by genes on autonomously replicating plasmids. This machinery may also enable the mobilization of plasmids that are non-conjugative (Ferreira et al., 2019).

Conjugation is regarded as an important process for the dissemination of genes conferring resistance to different classes of antibiotics and to metals. It supplies better defense from the surrounding environment and more efficient means of penetrating the host cell than transformation, while presenting a broader host range than bacteriophage transduction (von Wintersdorff et al., 2016). The conjugative plasmidome composition can be influenced by distinct intrinsic or external factors. Bacteria harboring multiple plasmids might conjugate more often than bacteria harboring a single plasmid. Thus, bacteria may transfer different plasmids on different occasions or simultaneously (Ferreira et al., 2019). The simultaneous transfer of different plasmids can involve genetic rearrangements. Moreover, multiple resistance genes are often co-localized on the same plasmid, allowing easier spread of multidrug resistance (von Wintersdorff et al., 2016). The presence or absence of a mobilization (relaxase and origin of transfer—oriT) and mating pore formation systems, allows the prediction of plasmid mobility. Through the conjugation process, the relaxase enzyme recognizes the oriT and cuts the plasmid DNA at a conserved nick site while the mating pore formation system produces the mating channel (Kesamang & Rahube, 2019).

1.7 Mobile genetic elements: Plasmids and incompatibility groups; Integrons

Plasmids are considered major drivers for antibiotic resistance dissemination. They can be horizontally transferred among different bacterial species from diverse taxonomic groups and enhance bacterial genetic diversity. Acquisition or deletion of adaptive genes from different phylogenetic origins promotes fast evolution and adaptation of bacterial hosts under worsening environmental conditions. Plasmids of the same Inc group cannot be disseminated in the same cell line. This competition for replication factors between plasmids assures a rapid outgrow of plasmids having growth advantages in the host cell. Inc group identification has been an important mechanism to track the propagation and evolution of different plasmids (Carattoli et al., 2005; Douarre et al., 2020; Kesamang & Rahube, 2019).

Integrons are capable of collecting environmental genes and incorporate them by site-specific recombination, mediated by the integron, integrase *IntI*1 (Singh et al., 2020) and can be incorporated into transposons and thereby become mobilized. These mobile genetic elements contain gene cassettes that can be incorporated or excised independently within the integron structure (Brolund & Larserics, 2013). Detection of integrons harboring antibiotic resistance genes has been described by several studies as a relevant pathway to identify certain environments as antibiotic and heavy metal resistance reservoirs (Henriques et al., 2006; Sarwar et al., 2021; Singh et al., 2020).

Table 3: Example of mobile genetic elements involved in resistance spread and respective characteristics. Source: (Brolund & Larserics 2013; Douarre et al., 2020; Henriques et al., 2006; Sarwar et al., 2021; Singh et al., 2020)

Mobile genetic elements	
Plasmids	**Integrons**
Extra-chromosomal fragments of DNA that replicate autonomously in a host cell	Bacterial hotspots of genotypic diversity capable of collecting environmental genes and incorporate them by site-specific recombination
Hold a conserved region, harboring genes required for plasmid replication, and a variable region regarding associated genes that might enable antimicrobial resistance or virulence traits	Recognized by the presence of an *intI* gene encoding an integrase, a recombination site (*attI*), and a promoter
Traditionally classified into several incompatibility (Inc) groups. Plasmids sharing the same replication or replicon origin can't stably coexist in a cell and are considered incompatible, whereas plasmids with different replication systems are compatible.	Contain gene cassettes that can be incorporated or excised independently within the integron structure. Frequently used as a genetic marker of anthropogenic pollutants studies
Plasmids from *Enterobacteriaceae* are the most studied and highly relevant in antibiotic resistance dissemination. Plasmids of replicons types IncHI2 and IncFIB are among the most commonly identified in *Enterobacteriaceae* and may coexist in the same cell	Most commonly found in proteobacteria

2 Objectives

This study aimed to compare *Escherichia coli* isolates from clinical, wastewater and surface water samples (n=52), in order to assess if resistance profiles were source-dependent. This study acknowledged the hypothesis that isolates with environmental origin, not specialized as pathogens, will likely have less mechanisms to resist antibiotics and to cause infection than clinical bacteria. Clinical and environmental phenotypic and genotypic analyses were performed to compare bacteria from the distinct origins.

A second objective was to understand if the rate and profile of antibiotic resistance gene transfer differed depending on the conjugation temperature. The knowledge of the specific conditions under which the resistant genes and plasmids are spread is of great importance to understand how this process is enhanced and how it can be stopped.

3 Methods

3.1 Bacterial strains

3.1.1 Bacteria and characterization

A group of 52 isolates presumably identified as *Escherichia coli* of clinical (n=34) and environmental (n=18) origin were selected for the study. The isolates were recovered between 2018 and 2019, by cultivation on Chromogenic Coliform Agar (CCA, VWR Chemicals) supplemented with cefotaxime (CTX, 2 µg/mL) incubated for 24 h at 37 °C. Blue colonies were isolated as presumable *E. coli* isolates. The isolates were cultured on PCA at 37 °C, observed after Gram-staining and preserved in Luria-Bertani (LB) medium supplemented with 10% glycerol (v/v) at -80ºC. The cultures were further processed for identification and phenotypic and genotypic characterization, as summarized in table 5.

3.1.2 Antibiotic Susceptibility testing

Antibiotic susceptibility was tested based on the disk diffusion method. A bacterial suspension was prepared with biomass from all the clinical and environmental isolates homogenized with 2 mL of saline solution (0.85 % NaCl), and the optical density (OD_{600}) was adjusted between 0.20-0.24 in each case. A swab impregnated with the bacterial suspension was used to inoculate the culture medium by streaking three times over the entire surface of a dried Muller-Hinton agar plate and incubated for 18 h at 37ºC with the antibiotic disks. The antibiotics tested were ciprofloxacin (CIP, 5 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), gentamicin (CN, 10 µg), meropenem (MEM, 10 µg), trimethoprim-sulphamethoxazole (SXT, 1.25/23.75 µg) and tetracycline (TE, 30 µg). The results were assessed (resistant, susceptible or intermediate susceptibility) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017).

3.1.3 Genotyping and gene detection

DNA extracts were obtained from fresh pure cultures by resuspending the bacterial colonies in 52 µl of nuclease-free water and heating for 10 min at 95 °C. The lysates were cooled in ice for 5 min and centrifuged at 14 000 rpm for 5 min, being the supernatant collected as the DNA extract to use as template for PCR reactions (Wiedmann-Al-Ahmad et al., 1994).

After DNA extraction all 52 isolates were subjected to PCR amplification of *lacZ* and 16S rRNA genes for their identification as putative *E. coli*.

The 16s rRNA gene PCR products were purified using GRS Genomic DNA Kit (GRiSP, Portugal), following manufacturer's instructions. After purification, these products were sequenced using the Sanger sequencing method. Phylogenetic analysis based on 16S rRNA gene sequences was conducted in the MEGA (molecular evolutionary genetics analysis) program, version 7, and compared with sequences available on the database EzBioCloud.

Isolates characterization was achieved by PCR screening of antibiotic resistant genes (*bla*$_{KPC,}$ *bla*$_{CTX\text{-}M}$), plasmid replicon type IncF and class 1 integron integrase (*intI-1*), using primers and conditions described in the table 4.

Table 4: Primers and conditions used in PCR analysis of different selected genes

Genes	Primers	Primer Sequence (5'-3')	Amplicon size	Protocol Source	Positive Control
lacZ	Big Z (forward)	GCA GCG TTG TTG CAG TGC	1350 bp	(Higgins et al., 2007)	*E. coli* A2FCC14
	Big Z (reverse)	GTC CCG CAG CGC AGA C			
16SrRNA	27F (forward)	AGA GTT TGA TCM TGG CTC AG	1500 bp	*(Weisburg et al., 1991)*	
	1492R (reverse)	CGG TTA CCT TGT TAC GAC TT			
bla$_{CTX\text{-}M}$	CTX-M (forward)	CRA TGT GCA GYA CCA GTA A	540 bp	(Weill et al., 2004)	
	CTX-M (reverse)	CGC RAT ATC RTT GGT GGT G			
bla$_{KPC}$	KPC (forward)	CAT TCA AGG GCT TTC TTG CTG C	538 bp	(Dallenne et al., 2010)	*K. pneumoniae* M138080_S1
	KPC (forward)	ACG ACG GCA TAG TCA TTT GC			
bla$_{SHV}$	*SHV* (forward)	TCG GGC CGC GTA GGC ATG AT	626 bp	*(Dipersio et al., 2005)*	*E. coli* A4FC7
	SHV (reverse)	AGC AGG GCG ACA ATC CCG CG			
Intl-1	Intl1_F (forward)	CCT CCC GCA CGA TGA TC	280 bp	*(Henriques et al., 2006)*	*E. coli* A2FCC14
	Intl1_R (reverse)	TCC ACG CAT CGT CAG GC			
IncF	Frep B (forward)	TGA TCG TTT AAG GAA TTT TG	270 bp	*(Carattoli et al., 2005)*	
	FrepB (reverse)	GAA GAT CAG TCA CAC CAT CC			
IncN	N (forward)	GTC TAA CGA GCT TAC CGA AG	270 bp		
	N (reverse)	GTT TCA ACT CTG CCA AGT TC			
FIB	FIB (forward)	GGA GTT CTG ACA CAC GAT TTT CTG	702 bp		
	FIB (reverse)	CTC CCG TCG CTT CAG GGC ATT			
RAPD	M13	GAG GGT GGC GGT TCT	Random sized amplicons	(da Silva et al., 2007)	

3.2 Conjugation assays

This conjugation assay protocol was based on a previous study (Ferreira et al., 2019), which tested the influence of selective stressors, such as metals or antibiotics, in the conjugation rate. In this work, were made the necessary adaptations. Conjugation assays were conducted with a bla_{KPC} positive *E. coli* strain IC2 (resistant to ceftazidime, cefotaxime and meropenem) as the donor and the azide resistant strain *E. coli* J53 as the recipient. Isolates growth was tested both on LA medium supplemented with ceftazidime (2 µg/ml) and LA supplemented with sodium azide (100 µg/ml). As expected, *E. coli* IC2 grew on LA supplemented with the antibiotic but not on the medium supplemented with sodium azide. Otherwise, *E. coli* J53 grew on LA supplemented with sodium azide due to its azide resistant phenotype but not on the medium supplemented with the antibiotic due to its lack of antibiotic resistance mechanisms.

LA medium supplemented with ceftazidime (2µg/ml) was used to determine the number of viable donors. After mating, LA medium supplemented with ceftazidime (2 µg/ml) and sodium azide (100 µg/ml) was used to analyze the number of transconjugants by decimal dilutions method and to further select transconjugant colonies.

Donor and recipient strains were grown in 10 ml Luria-Bertani (LB) broth for 4 h at 37ºC, until the late-exponential growth phase, corresponding to an optical density between 0.6 and 1.0 (OD600). Both cultures were mixed in a 1:1 ratio based on optical density measurement, centrifuged at 10 000 rpm for 5 min, and suspended in fresh LB medium, and incubated for 20 h. Conjugation was examined at different mating temperatures to assess the influence of temperature on the conjugation rate. Donor and recipient cultures as described above were mated at temperatures of 25, 28, 35, and 40ºC, each time in triplicate. Conjugation efficiency rate was calculated based on the ratio of the number of presumable transconjugant colonies (able to grow on medium with azide and ceftazidime) and the number of viable donor colonies. About 10% of randomly selected putative transconjugant colonies (strain J53 harboring bla_{KPC} genes) were cultured on LA medium supplemented with sodium azide and ceftazidime and cryopreserved. DNA was extracted from each of these cultures. The authenticity of 35 putative transconjugants recovered in each of the four temperatures tested, was genotyped by random amplified polymorphic DNA (RAPD) to confirm the presence of the genotype of the recipient strain (Ferreira et al., 2019). Confirmed authentic transconjugants were screened for the presence of the bla_{KPC} gene and the plasmid incompatibility groups Inc N and FIB, by PCR. FIB positive transconjugants were further characterized for the presence of the integrase and bla_{SHV} genes, both known to be present in the same sequencing contig as the replicon type FIB. Primers and conditions used in RAPD, bla_{KPC}, bla_{SHV}, IncN, FIB and Integrase PCR amplifications are presented in table 4.

3.3 Statistical analysis

Statistical analysis involved descriptive statistics (absolute and relative frequencies, means, and their respective standard deviations) and inferential statistics. In this analysis, the prevalence of phenotypic resistance to antibiotics and antibiotic resistance genes, as their possible differences between clinical and environmental isolates were accessed by Fisher's and Chi-square independence tests. The differences of multidrug resistant isolates and isolates identified as *E. coli* by 16S rRNA sequencing, between isolates source, was accessed by the Binomial test. The influence of mating temperature in the conjugation rate was analyzed by Spearman correlation coefficient. The significance level for rejecting the null hypothesis was set at $(\alpha) \leq 0.05$. Statistical analysis was performed using SPSS (Statistical Package for the Social Sciences) version 27 for Windows.

4 Results / Discussion

4.1 Isolates identification

The collection of cefotaxime resistant *Enterobacteriaceae* strains available for this study included 18 of wastewater and surface water environments, herein designated environmental and 34 from urinary tract infections, herein designated clinical. The first part of the work consisted of the identification to the genus or species levels. In a first trial isolates were screened for the presence of the gene *lacZ*, expected to occur in *E. coli*, but not in most of the species of the family. After this dichotomic analysis for the *lacZ* gene, selected positive strains and all negative strains were analysed based on 16S rRNA genes sequencing. Curiously, a clinical isolate (IC23), later identified as *E. coli* tested negative for the presence of *lacZ*. The query of the EzBioCloud 16S rRNA gene sequences database and considering sequence identity values above 98% permitted the identification of 34 isolates as member of the *Escherichia* genus, comprising *Escherichia* coli*,* *Escherichia fergusonii*, and *Escherichia marmotae* species. Some isolates (n=16) were identified as member of the Shigella genus, comprising *Shigella dysenteriae* and *Shigella flexneri*, which belong to the same phylogenetic group as *Escherichia coli*. Two environmental isolates were identified as *Citrobacter portucalensis* and *Citrobacter freundii.* Identified strains are presented in figure 6.

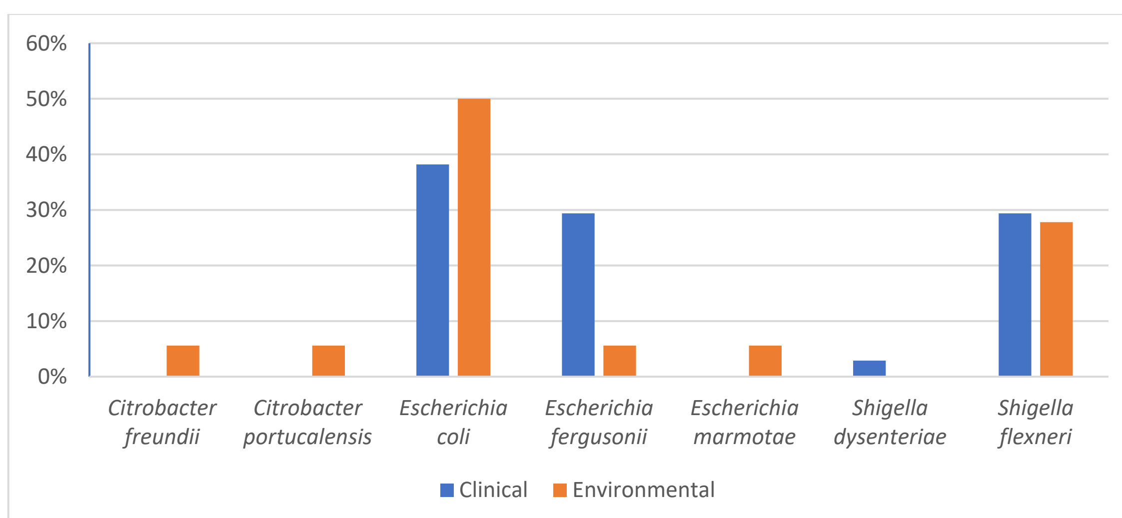
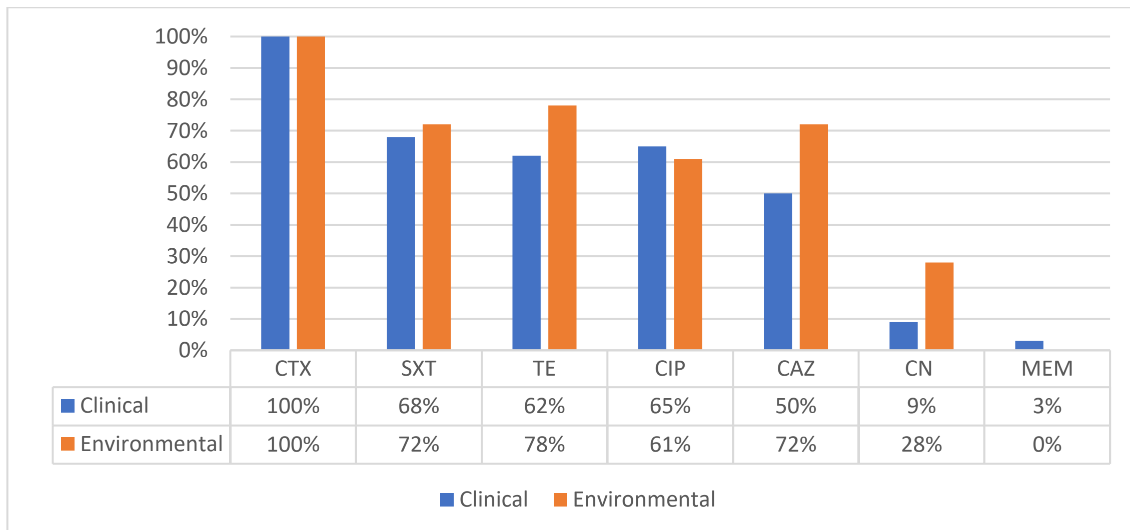


Figure 6: Percentage values of species identified based on 16S rRNA gene sequence analysis. A total of 52 isolates were identified into species level (18 environmental and 34 clinical). The difference between the percentage of identified Escherichia coli *(38% vs50%) and* Shigella flexneri *(29% vs 28%) in clinical and environmental isolates was not statistically significant, Binomial test, respectively p= 0.229 and p= 0.481. However, the percentage of* Escherichia fergusonii *(29% vs 5.6%) was statistically significantly higher among clinical isolates, Binomial test, p < 0.001.*

4.2 Phenotypic characterization

Resistance was tested against seven different antibiotics. As part of the previous experimental design, all tested isolates were resistant to cefotaxime (CTX), elected as the common trait between clinical and environmental isolates, which would facilitate further comparative analysis. More than half of the isolates were resistant to ciprofloxacin (CIP), tetracycline (TE), trimethoprim-sulphamethoxazole (SXT), and ceftazidime (CAZ). In contrast, less than half of the isolates was resistant to gentamicin (CN) and meropenem (MEM). Indeed, a single clinical isolate (*E. coli* IC2) was observed to be resistant to carbapenems, specifically to meropenem, regarded as a last-resort drug to treat multi-drug resistant bacterial infections.

Refuting the hypothesis of the study, no statistically significant differences were observed for the antibiotic resistance prevalence between clinical and environmental isolates, for any of the seven antibiotics (Figure 7).



	CTX	SXT	TE	CIP	CAZ	CN	MEM
Clinical	100%	68%	62%	65%	50%	9%	3%
Environmental	100%	72%	78%	61%	72%	28%	0%

Figure 7 Percentage values of resistant clinical and environmental isolates for each tested antibiotic. A total of 52 isolates were tested for antibiotic resistance (18 environmental and 34 clinical). The prevalence of resistance phenotypes and possible differences between isolates from different sources (clinical and environmental) were accessed using fisher's test for TE, SXT, MEM and CN (which presented resistant and susceptible isolates.) and chi-square of independence for CIP and CAZ (which presented isolates resistant, susceptible and with intermediate susceptibility). Ciprofloxacin (CIP) χ^2 (2) = 0.237, p = 1.000; ceftazidime (CAZ) χ2 (2) = 2.383, p = 0.371; tetracycline (TE) Fisher's test, p= 0.354; trimethoprim-sulphamethoxazole (SXT) Fisher's test, p = 1.000; meropenem (MEM) Fisher's test, p = 0.108.; gentamicin (CN) Fisher's test, p = 0.108.

Isolates resistant to at least one antibiotic belonging to three or more distinct antibiotic classes were considered multidrug-resistant. Considering six antibiotic classes - tetracyclines, quinolones, aminoglycosides, sulphonamides, cephalosporins, carbapenems - it was observed that cefotaxime resistant isolates were mostly multidrug-resistant (71% of the isolates). The difference between the percentage of multidrug-resistant isolates among clinical and environmental isolates was not statistically significant (70.6% vs 72.2%), Binomial test, p = .481.

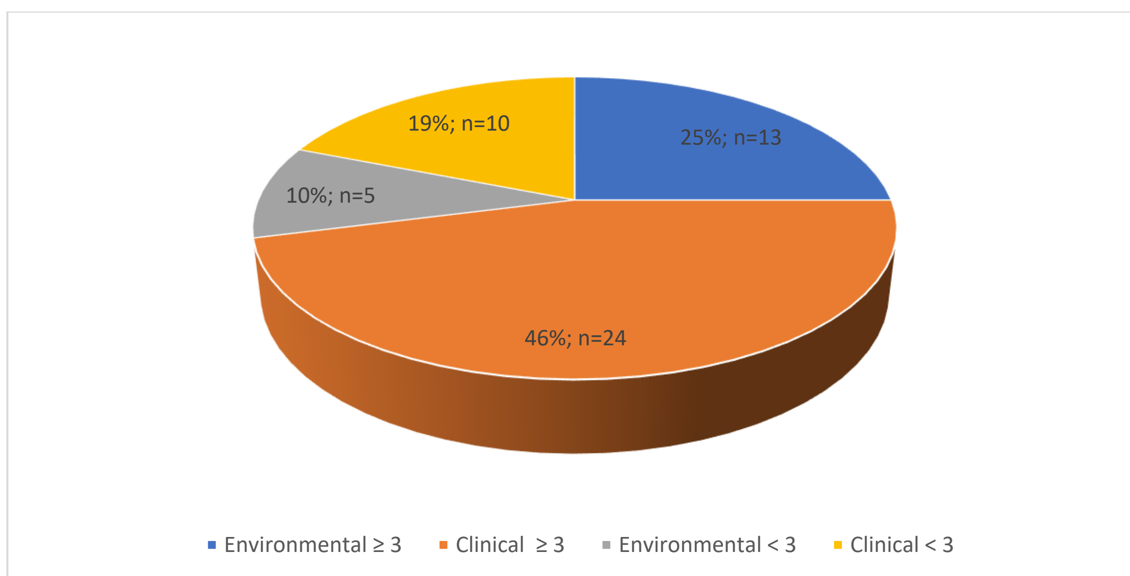
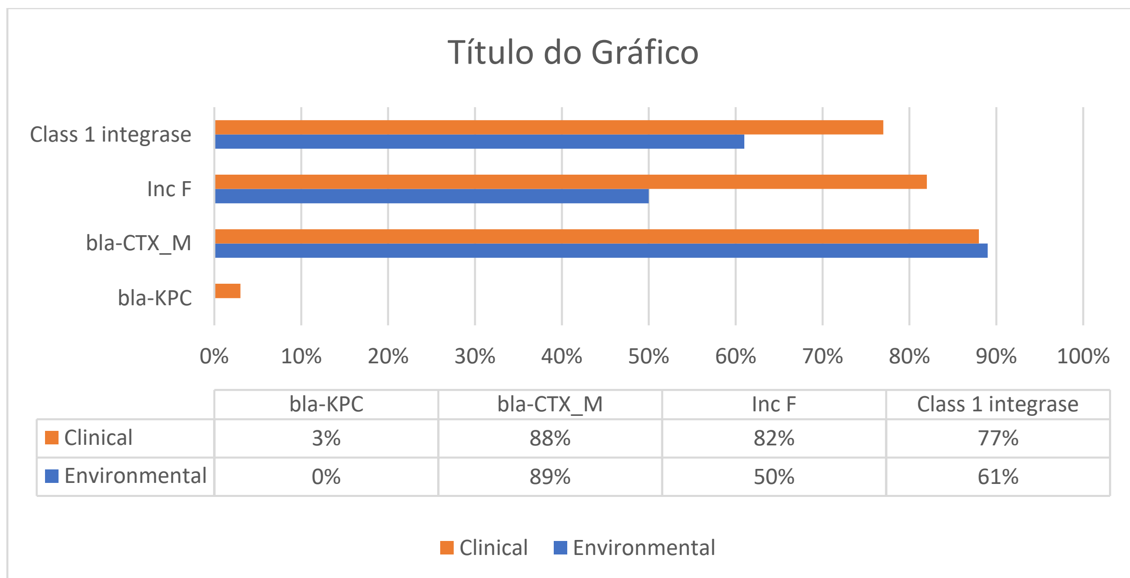


Figure 8: Percentage of multidrug-resistant isolates. Of a total of 52 tested isolates (18 environmental and 34 clinical), 37 isolates were considered multidrug-resistance (13 environmental and 24 clinical).

4.3 Genotypic characterization

The isolates were characterized for the presence of β-lactamase encoding genes (*bla*$_{KPC}$; *bla*$_{CTX-M}$), plasmids incompatibility group IncF, and class 1 integron integrase. Cefotaxime resistance could be explained in most of the isolates due to the presence of the *bla*$_{CTX}$ gene (89%). In contrast, the *bla*$_{KPC}$ gene was detected in a single clinical isolate (*E. coli* IC2). The presence of this gene in this specific isolate could be expected as it was the only one previously presenting meropenem phenotypic resistance. The class 1 integron integrase gene was also detected in most isolates (71%), 89% of these were multidrug resistant. An identical percentage of isolates yielded the incF plasmid replicon type (71%). Of these incF positive isolates 76% harbored also the gene intI1 and 70% were multidrug resistant.



	bla-KPC	bla-CTX_M	Inc F	Class 1 integrase
Clinical	3%	88%	82%	77%
Environmental	0%	89%	50%	61%

Figure 9: Prevalence values of selected resistant genes among tested Isolates from clinical and environmental settings. PCR detection of selected genes was conducted on all 52 isolates (18 environmental and 34 clinical). Except for the incF plasmid replicon type, there were no significant differences of prevalence values between clinical and environmental isolates of bla-genes (bla_{KPC}*,* $bla_{CTX\text{-}M}$*), and class-1 integrase.* bla_{KPC} *Fisher's test p = 1.000;* bla_{CTX} *Fisher's test p = 1.000; IncF Fisher test p = .024; class1 integrase Fisher's test p = 1.000. In contrast, a significantly higher proportion of IncF plasmid replicon type was detected among clinical (82.4%) than among environmental isolates (50.0%).*

An overview of the phenotypic and genotypic characterization of the isolates is presented in table 5. This study acknowledged the hypothesis that environmental isolates, not specialized as pathogens, would likely have less mechanisms to resist antibiotics and to cause infection than clinical bacteria. The latter are subjected to higher selective pressures and stimulated to be specialized in surviving host defense. However, in general no significant differences were observed, except for incF plasmid replicon type.

Table 5: Antibiotic resistance phenotypes and genotypes of the Enterobacteriaceae *isolates with clinic and environmental origin*

			Antibioitc resistance genes		Mobile gentic elements		Antibiotic resistance profile							
			β-lactamases		Plasmid replicon	Class 1 integrase	Quinolone	Cefalosporins		Aminoglycosides	Carbapenems	Sulfonamides	Tetracycline	
Isolates ID	**Sample origin**	**Species**	***bla*$_{CTX-M}$**	***bla*$_{KPC}$**	**incF**	***intI1***	**CIP**	**CAZ**	**CTX**	**CN**	**MEM**	**SXT**	**TE**	**MDR***
IC2	clinic	*Escherichia coli*	-	+	+	+	S	R	R	S	R	S	S	2
IC21	clinic	*Escherichia fergusonii*	+	-	+	+	I	I	R	S	S	R	R	3
IC22	clinic	*Escherichia fergusonii*	+	-	-	-	S	I	R	S	S	S	S	1
IC23	clinic	*Escherichia coli*	-	-	+	+	R	R	R	S	S	S	R	3
IC24	clinic	*Escherichia coli*	+	-	+	+	R	I	R	S	S	R	R	4
IC25	clinic	*Escherichia fergusonii*	+	-	+	+	R	I	R	S	S	S	R	3
IC26	clinic	*Escherichia coli*	+	-	+	+	R	R	R	S	S	R	S	3
IC27	clinic	*Escherichia coli*	+	-	+	+	R	R	R	S	S	R	R	4
IC28	clinic	*Shigella flexneri*	+	-	+	+	S	R	R	S	S	S	R	2
IC29	clinic	*Escherichia coli*	+	-	+	+	R	R	R	S	S	R	R	4
IC30	clinic	*Escherichia coli*	+	-	+	+	S	R	R	R	S	R	R	4
IC31	clinic	*Escherichia coli*	+	-	-	+	R	R	R	S	S	R	R	4
IC32	clinic	*Shigella flexneri*	+	-	+	-	R	I	R	S	S	R	R	4
IC33	clinic	*Shigella dysenteriae*	+	-	+	+	R	I	R	S	S	R	R	4
IC34	clinic	*Escherichia fergusonii*	+	-	+	-	S	S	R	S	S	R	S	2
IC35	clinic	*Escherichia fergusonii*	+	-	+	+	S	I	R	S	S	R	S	2
IC36	clinic	*Shigella flexneri*	+	-	-	+	R	S	R	S	S	R	R	4
IC37	clinic	*Escherichia fergusonii*	+	-	+	-	R	R	R	S	S	S	S	2
IC38	clinic	*Shigella flexneri*	+	-	-	-	S	S	R	S	S	S	S	1
IC39	clinic	*Shigella flexneri*	+	-	+	+	R	R	R	S	S	R	R	4
IC40	clinic	*Escherichia fergusonii*	+	-	+	+	S	R	R	S	S	R	R	3
IC41	clinic	*Shigella flexneri*	+	-	+	+	R	I	R	S	S	R	S	3
IC42	clinic	*Escherichia fergusonii*	+	-	+	+	R	R	R	S	S	R	R	4
IC43	clinic	*Escherichia coli*	+	-	+	+	R	R	R	S	S	R	S	3
IC44	clinic	*Shigella flexneri*	-	-	+	-	S	S	R	S	S	S	S	1
IC45	clinic	*Shigella flexneri*	+	-	+	+	R	S	R	S	S	R	R	4
IC46	clinic	*Escherichia coli*	+	-	-	+	R	R	R	R	S	R	S	4
IC47	clinic	*Escherichia coli*	+	-	+	+	R	I	R	S	S	R	R	4
IC48	clinic	*Escherichia fergusonii*	-	-	+	+	S	S	R	S	S	S	S	1
IC49	clinic	*Escherichia coli*	+	-	+	-	R	R	R	R	S	S	R	4
IC50	clinic	*Escherichia fergusonii*	+	-	+	+	R	S	R	S	S	R	R	4
IC51	clinic	*Shigella flexneri*	+	-	-	+	R	I	R	S	S	R	S	3
IC52	clinic	*Escherichia coli*	+	-	+	-	S	R	R	S	S	S	R	2
IC53	clinic	*Escherichia coli*	+	-	+	+	R	R	R	S	S	R	R	4
A1	environmental	*Citrobacter portucalensis*	-	-	-	-	S	R	R	S	S	S	R	2
E1	environmental	*Shigella flexneri*	+	-	-	+	S	I	R	R	S	R	S	3
E2	environmental	*Shigella flexneri*	+	-	-	+	S	S	R	S	S	R	R	3
E3	environmental	*Escherichia coli*	+	-	+	-	R	R	R	S	S	S	S	2
R1	environmental	*Shigella flexneri*	+	-	-	+	S	S	R	S	S	R	R	3
R2	environmental	*Escherichia coli*	+	-	+	+	R	R	R	R	S	R	R	5
V1	environmental	*Shigella flexneri*	+	-	-	-	S	I	R	S	S	S	R	2
V2	environmental	*Escherichia coli*	+	-	-	-	R	R	R	S	S	R	S	3
V3	environmental	*Escherichia coli*	+	-	+	+	R	R	R	R	S	R	R	5
V4	environmental	*Escherichia coli*	+	-	+	+	R	R	R	R	S	R	R	5
V5	environmental	*Escherichia coli*	+	-	-	+	R	R	R	S	S	R	R	4
V6	environmental	*Escherichia marmotae*	+	-	-	-	R	I	R	S	S	R	R	4
V7	environmental	*Citrobacter freundii*	-	-	-	+	R	R	R	R	S	R	R	5
V8	environmental	*Shigella flexneri*	+	-	+	+	I	R	R	S	S	R	R	3
V9	environmental	*Escherichia coli*	+	-	+	+	R	R	R	S	S	R	R	4
Z1	environmental	*Escherichia coli*	+	-	+	-	S	R	R	S	S	S	R	2
Z2	environmental	*Escherichia coli*	+	-	+	+	R	R	R	S	S	R	R	4
Z3	environmental	*Escherichia coli*	+	-	+	-	R	R	R	S	S	S	S	2

4.4 Effect of temperature on conjugation rate and transconjugant profile

Given the observation that the *incF* plasmid replicon type was more frequent among clinical than among environmental isolates, it was proposed the hypothesis that this plasmid could be preferentially mobilized at higher temperatures as those observed in the human body. To test this hypothesis was selected strain *E. coli* IC2 that harbored *intI1*, *incF* and the bla_{KPC} gene. This strain had conjugative capacity, with the transfer of the bla_{KPC} gene harbored in an *incN* plasmid type, as tested in parallel studies. Conjugation assays were performed under different matting temperature conditions (25, 28, 35, and 40ºC), to assess the influence of mating temperature on the conjugation rate, and which genotypes and phenotypes were acquired by the recipient strain.

In total 12 conjugation assays (triplicates at four distinct temperatures) were performed. Within each temperature the conjugation rate varied between 3.5×10^{-3} and 9.1×10^{-3}, suggesting the high reproducibility of the assays. The verification of the authenticity of presumable transconjugants was made based on a random sampling procedure, with a total of 140 colonies recovered on the medium with ceftazidime and azide being testes - 35 collected from each temperature assayed. At the highest temperature tested (40ºC) all 35 selected transconjugants were authentic. At the temperatures of 35ºC and 28ºC were observed 4/35 false-positives and 8/35 at the lowest temperature tested (25ºC). These values were used to estimate the effective conjugation rates. The whole procedure and the number of viable donors and transconjugants replicate means and respective standard deviations are summarized in table 6.

Table 6: Number of viable donors, transconjugants replicate means, percentage of RAPD validated transconjugants, conjugation rate and respective standard deviations. The correlation between the conjugation rate and temperature is not statistically significant; *Spearman correlation coefficient* (p = 0.410)

Temperature of matting	**Conjugation replicate**	**Donor Bacteria (CFU/ml)**	**SD**	**Mean Donor (CFU/mL)**	**SD**	**Presumable Tranconjugants colonies (CFU/mL)**	**SD**	**Mean transconjugants (CFU/mL)**	**SD**	**% RAPD Confirmed transconjugants**	**Validated transconjugants (CFU/mL)**	**Mean Validated transconjugants (CFU/mL)**	**SD**	**Validated Transconjugants /Viable Donor**	**Mean Transconjugants/Viable Donor**	**SD**	**conjugation rate (%)**	**Mean conjugation rate (%)**	**SD**
	1	$3.8\ 10^{8}$	$8.0\ 10^{7}$	$3.3\ 10^{8}$	$3.3\ 10^{7}$	$1.0\ 10^{4}$	$1.1\ 10^{3}$	$1.3\ 10^{4}$	$1.7\ 10^{3}$	78	$7.9\ 10^{3}$	$1.1\ 10^{4}$	$1.1\ 10^{4}$	$2.1\ 10^{-5}$	$3.5\ 10^{-5}$	$3.9\ 10^{-5}$	$2.1\ 10^{-3}$	$3.5\ 10^{-3}$	$3.9\ 10^{-3}$
25°C	2	$2.8\ 10^{8}$	$5.5\ 10^{7}$			$2.2\ 10^{4}$	$4.0\ 10^{3}$			100	$2.2\ 10^{4}$			$7.9\ 10^{-5}$			$7.9\ 10^{-3}$		
	3	$3.2\ 10^{8}$	$1.5\ 10^{7}$			$7.1\ 10^{4}$	$9.2\ 10^{2}$			25	$1.7\ 10^{3}$			$5.5\ 10^{-6}$			$5.5\ 10^{-4}$		
	1	$3.8\ 10^{8}$	$8.0\ 10^{7}$	$3.3\ 10^{8}$	$3.3\ 10^{7}$	$1.2\ 10^{4}$	$3.5\ 10^{3}$	$1.5\ 10^{4}$	$4.6\ 10^{2}$	92	$1.1\ 10^{4}$	$1.3\ 10^{4}$	$1.6\ 10^{3}$	$2.9\ 10^{-5}$	$4.1\ 10^{-5}$	$9.9\ 10^{-6}$	$2.9\ 10^{-3}$	$4.1\ 10^{-3}$	$9.9\ 10^{-4}$
28°C	2	$2.8\ 10^{8}$	$5.5\ 10^{7}$			$1.3\ 10^{4}$	$3.7\ 10^{3}$			100	$1.4\ 10^{4}$			$4.8\ 10^{-5}$			$4.8\ 10^{-3}$		
	3	$3.2\ 10^{8}$	$1.5\ 10^{7}$			$1.9\ 10^{4}$	$2.8\ 10^{3}$			75	$1.4\ 10^{4}$			$4.5\ 10^{-5}$			$4.5\ 10^{-3}$		
	1	$3.8\ 10^{8}$	$8.0\ 10^{7}$	$3.3\ 10^{8}$	$3.3\ 10^{7}$	$3.0\ 10^{4}$	0	$3.1\ 10^{4}$	$4.0\ 10^{3}$	71	$2.1\ 10^{4}$	$2.9\ 10^{4}$	$8.0\ 10^{3}$	$5.5\ 10^{-5}$	$9.1\ 10^{-5}$	$3.8\ 10^{-5}$	$5.5\ 10^{-3}$	$9.1\ 10^{-3}$	$3.8\ 10^{-3}$
35°C	2	$2.8\ 10^{8}$	$5.5\ 10^{7}$			$3.7\ 10^{4}$	$7.7\ 10^{3}$			100	$3.7\ 10^{4}$			$1.3\ 10^{-4}$			$1.3\ 10^{-2}$		
	3	$3.2\ 10^{8}$	$1.5\ 10^{7}$			$2.8\ 10^{4}$	$2.0\ 10^{3}$			100	$2.8\ 10^{4}$			$8.8\ 10^{-5}$			$8.8\ 10^{-3}$		
	1	$3.8\ 10^{8}$	$8.0\ 10^{7}$	$3.3\ 10^{8}$	$3.3\ 10^{7}$	$1.6\ 10^{4}$	$9.1\ 10^{3}$	$1.7\ 10^{4}$	$7.8\ 10^{3}$	100	$1.6\ 10^{4}$	$1.8\ 10^{4}$	$7.8\ 10^{3}$	$4.1\ 10^{-5}$	$5.6\ 10^{-5}$	$3.2\ 10^{-5}$	$4.1\ 10^{-3}$	$5.6\ 10^{-3}$	$3.2\ 10^{-3}$
40°C	2	$2.8\ 10^{8}$	$5.5\ 10^{7}$			$2.6\ 10^{4}$	$1.7\ 10^{4}$			100	$2.6\ 10^{4}$			$9.2\ 10^{-5}$			$9.2\ 10^{-3}$		
	3	$3.2\ 10^{8}$	$1.5\ 10^{7}$			$1.1\ 10^{4}$	$1.0\ 10^{3}$			100	$1.1\ 10^{4}$			$3.4\ 10^{-5}$			$3.4\ 10^{-3}$		

SD, standard deviation, CFU/mL, colony forming unit per mL of bacterial suspension

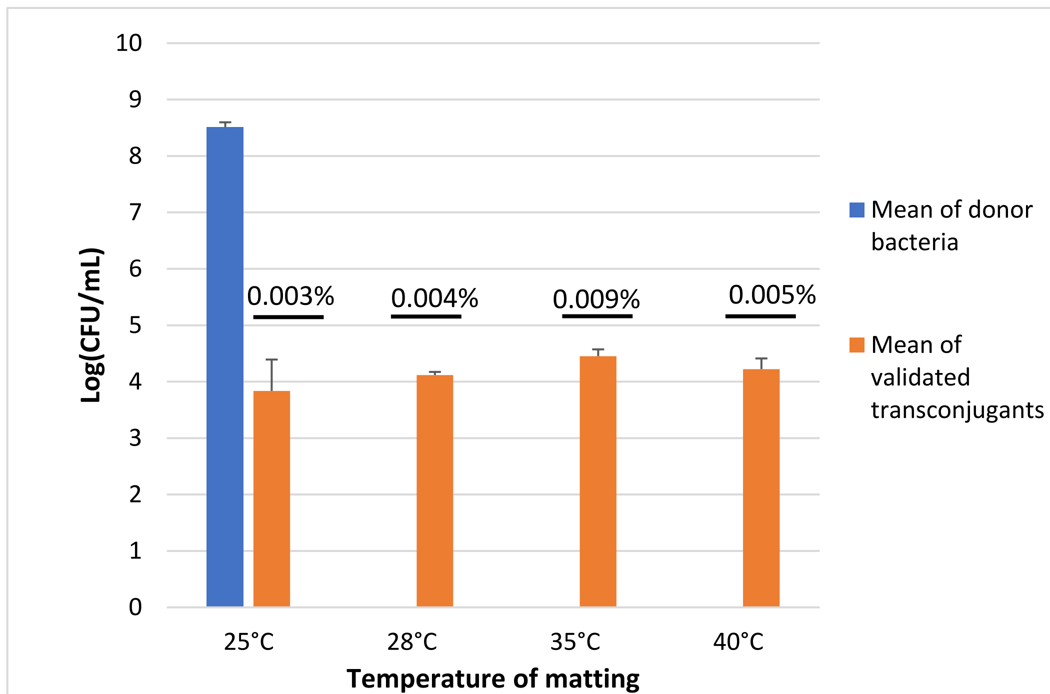


Figure 10: Validated transconjugants counts mean, viable donor counts mean, and validated conjugation rate at each temperature. The hypothesis tested in this section of the work was refuted as the conjugation rates were not significantly different at distinct temperatures (25,28,35,40°C) and the correlation between the conjugation rate and temperature is not statistically significant ($p > 0.05$)

Once refuted the hypothesis that higher temperatures might be associated with higher conjugation rates, it was raised another hypothesis, which drove the next part of the work. It was hypothesized that the profile of transconjugants could vary with temperature. Hence, the 124 confirmed transconjugants were further examined for the presence of the bla_{KPC} gene and IncN / FIB plasmid replicons types. This analysis revealed that all transconjugants were bla_{KPC} and *incN* positive, suggesting the high conjugative capacity of that plasmid. Parallel ongoing studies informed that the draft genome sequence of *E. coli* IC2 contained a contig where the plasmid replicon type Inc FIB, the bla_{SHV} and *Intl1* genes were present. This information, paved background to further characterize the genetic profile of the transconjugants, based on those genetic determinants. While these gene determinants were not detected in all transconjugants, all that displayed the plasmid replicon type FIB, has also the bla_{SHV} and *intl1* genes. The FIB replicon type and associated genes was more frequently detected among transconjugants obtained at higher mating temperatures. At 25ºC and 28ºC, it was observed that a single transconjugant (1/27 and 1/31, respectively) harbored FIB plasmids. This result contrasted with what was observed at higher temperatures, with 12/31 and 20/35 transconjugants displaying the FIB replicon type, at 35ºC and 40 ºC, respectively. These results support the hypothesis being tested, suggesting that the dissemination of FIB plasmids may be favored in high temperature environment as the human body, while the transfer of incN may occur in a broader environmental context.

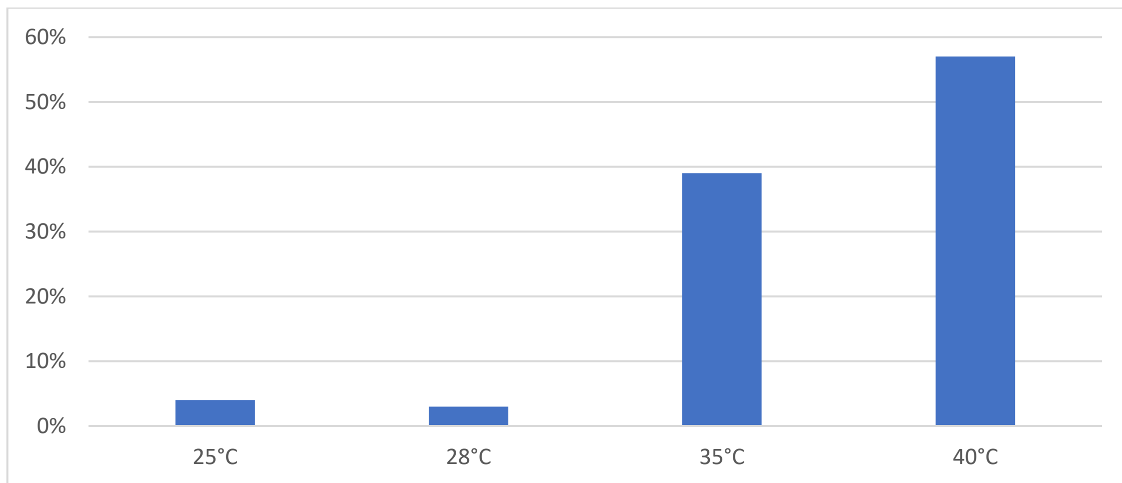


Figure 11: Percentage of replicon type FIB positive transconjugants harboring bla_{SHV} *and* intI1 *genes. PCR detection was conducted on all 124 confirmed transconjugants form every matting temperature (27 from 25°C, 31 from 28°C and 35°C, 35 from 40°C). There was a significantly higher proportion of positive results at 40°C and negatives at 25°C and 28°C, $\chi 2$ (3) = 34,272, p = 0.001.*

As an opportunistic human pathogen, members of the species *E. coli* are distributed throughout different clinical and environmental reservoirs and present in mammals' intestinal tracts. Bacterial infection might enhance the tendency of bacterial conjugation, to transfer genetic traits between bacteria inside the host organism. This fact might explain the larger transmission of genetic material at 40ºC, typical host organism fever temperature, during bacterial infection.

5 Conclusion

The rapid spread of antibiotic resistance among human pathogenic bacteria has been a global public health problem. Several studies, over the past decades, revealed the fast emergence of resistant bacteria, both in clinical and environmental settings (Brolund & Larserics Digital, 2013; Dipersio et al., 2005; Ferreira et al., 2019; Manaia et al., 2018; Paterson et al., 2003; Pfeifer et al., 2010; Rocha et al., 2022; Sun et al., 2019; von Wintersdorff et al., 2016). Thus, some studies comparing both isolate sources presented significantly higher resistance percentage values among clinical isolates (testing phenotypic and genotypic antibiotic resistance and other virulence traits) (Fagade et al., 2010; I.U. Rathnayake et al., 2012; Sivaraj et al., 2012). Supporting the hypothesis that isolates with an environmental origin, not specialized as pathogens, will likely have fewer mechanisms to resist antibiotics and to cause infection than clinical bacteria, which motivated the first part of the work.

More than half of the cefotaxime resistant isolates were also resistant to ciprofloxacin, tetracycline, trimethoprim-sulphamethoxazole, or ceftazidime. Comparatively lower resistance prevalence was observed for gentamicin and meropenem. The antibiotic resistance phenotypes did not evidence statistically significant different prevalence values between clinical and environmental isolates. This result was confirmed by the observation of non-significantly different percentage of multidrug-resistant isolates among clinical and environmental isolates (71% in both cases). Among the analyzed genetic determinants, it was observed that the plasmid replicon type IncF was significantly more frequent in clinical (82%) than in environmental isolates (50%). These differences were not observed for the other genes - *bla*-genes (*bla*$_{KPC}$, *bla*$_{CTX}$), and class1 integron integrase. Such a result hinted that different plasmids my respond differently to external conditions, such as temperature.

Horizontal gene transfer, in particular through conjugation, is considered the main mechanism for antibiotic resistance spread in the environment (von Wintersdorff et al., 2016) It is also believed that environmental stressors are capable of enhancing and shaping genetic transfer efficiency (Berendonk et al., 2015; Rizzo et al., 2013). Ferreira et al., 2019, analyzed the conjugative plasmidome of a hospital effluent multidrug resistant *Escherichia coli* strain under different conditions, such as the presence of tellurite or arsenite. Concluding that conjugation efficiency was not significantly different between stress and stress-free conditions, however, arsenite presence favored the transfer of plasmid replicon type FIA/FIB. Such results suggests that conjugation conditions might enhance the transfer of specific genetic traits (Ferreira et al., 2019). This conclusion motivated the second part of the study that involved clinical Isolate *E. coli* IC2 that harbored *bla*$_{KPC}$ gene, exhibited phenotypic meropenem resistance and based on parallel genome analysis it was known to contain a FIB plasmid with the *intI1* and *bla*$_{SHV}$ genes. While different mating temperatures did not significantly influence the conjugation rate, transconjugation profile was suggested to be affected. The FIB plasmid replicon type, along with *bla*$_{SHV}$ and *intI1* genes, were more frequently transferred at the highest temperatures tested. In contrast, the IncN replicon type plasmid and the associated *bla*$_{KPC}$ gene transfer seemed to be independent of the environmental temperature.

6 Future work

Characterization and identification of a higher sample of clinical and environmental isolates and detection of more specific genes could unveil higher statistically significant differences between clinical or environmental isolates sources.

Conjugation could be examined under different stress conditions such as the presence/absence of peroxide and other compounds (metals, antibiotic pressures), and UV radiation in order to assess if other condition beside temperature may influence the transconjugants profile.

Other methods such as Pulse Field Gel Electrophoresis (PFGE) or long-read sequencing may be informative to assess the genetic linkage and accessory genes that can be transferred and acquired through conjugation, under different conditions.

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