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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Industrial e Ambiental, realizada sob a orientação científica da Doutora Isabel da Silva Henriques, Investigadora Auxiliar do CESAM, Universidade de Aveiro e co-orientação do Professor Doutor António Carlos Matias Correia, Professor Catedrático do Departamento de Biologia, Universidade de Aveiro.

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“Em cada um de nós há um Egipto e um Faraó e um Moisés e uma Liberdade numa Terra Prometida. E, a cada momento no tempo, há uma oportunidade para outro Êxodo. (…) Mas a Liberdade e a Terra Prometida não são elementos estáticos que ficam ali à espera. São conquistas tuas, que podes criar a qualquer momento, em qualquer coisa que fizeres, só por te livrares de quem eras na véspera.”

Lubavitcher Rebbe

o júri

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palavras-chave

Resistência a antibióticos, contaminação fecal, gaivotas, Escherichia coli, Ilhas das Berlengas

resumo

A descoberta e produção de antibióticos foi um grande avanço para a medicina na primeira metade do século passado. No entanto, as bactérias adaptaram-se rapidamente desenvolvendo mecanismos de resistência aos antibióticos cuja disseminação é facilitada pela transferência horizontal de genes. Hoje em dia a resistência a antibióticos constitui um problema de saúde pública sendo detectada não só a nível clínico, mas também em ambientes naturais, com particular destaque para os ambientes aquáticos.

As ilhas das Berlengas são uma reserva natural. No entanto tem sido detectada poluição associada a contaminação fecal na água da praia. Estudos anteriores concluíram que a principal origem desta contaminação são as fezes de gaivotas existentes na ilha.

O objectivo principal deste estudo foi analisar o perfil de resistência a antibióticos de isolados de Escherichia coli obtidos da água da praia da ilha das Berlengas, de fezes de gaivotas e do único efluente de águas residuais de origem humana existente na ilha. Com isto, pretende-se avaliar o risco para a saúde pública da contaminação fecal da água e confirmar a origem dessa poluição. Foi também objectivo deste trabalho identificar marcadores associados a resistência a antibióticos que contribuam para descriminar as fontes de poluição fecal.

Neste sentido procedeu-se à classificação dos 414 isolados de E. coli das diferentes fontes de acordo com os principais grupos filogenéticos (A, B1, B2 e D): mais de 70% dos isolados pertenciam aos grupos A e B1 geralmente associados a estirpes comensais. Nas três fontes estudadas existem isolados do grupo A, B1 e D. Verificaram-se isolados do grupo B2 apenas no efluente (10,1%) e em fezes (3,9%).

Foi avaliada a susceptibilidade a antibióticos usando o método de difusão em disco. Globalmente registaram-se para a ilha das Berlengas elevadas percentagens de resistência aos antibióticos testados, com prevalência a nível das penicilinas, aminoglicosídeos e tetraciclinas. A resistência a cefalosporinas de 3ª geração, ao imipenemo e ciprofloxacina foi rara e mais frequente em isolados do efluente. Registou-se uma elevada taxa de isolados multiresistentes (cerca de 30%). Foram pesquisadas as bases genéticas para os fenótipos obtidos, sendo bla_{TEM}, tet(A) e sul2 os genes mais frequentemente detectados. Verificou-se a ocorrência de $bla_{CTX\text{-}M\text{-}1}$ num isolado de água e $bla_{CMY\text{-}2}$ num de fezes. O contexto genético determinado para estes genes foi idêntico ao previamente descrito para isolados clínicos.

Obtiveram-se diferenças significativas entre o padrão de resistência da água e do efluente. Além disso, alguns dos fenótipos e genótipos detectados em isolados de água ocorreram apenas numa das fontes: fezes (em maior número) ou efluente. Para a utilização destes fenótipos e genótipos como marcadores seriam necessários mais estudos. Este estudo mostra que a poluição fecal associada a fezes de gaivotas, embora geralmente considerada menos grave que a associada a fezes de humanos, pode constituir um risco para a saúde pública.

(texto escrito segundo o antigo acordo ortográfico)

keywords

Antibiotic resistance, fecal pollution, seagull, Escherichia coli, , Ilhas das Berlengas

abstract

The discovery and production of antibiotics was a major breakthrough for medicine in the first half of the last century. However, bacteria have adapted quickly through the development of antibiotic resistance mechanisms that spread easily by horizontal gene transfer. The acquisition and dissemination of resistance were promoted by the intensive (mis)use of antibiotics.

Nowadays, antibiotic resistance is a public health problem and is found not only in clinical isolates but also in natural environments with particular emphasis for the aquatic ones.

The Berlengas Islands are a natural reserve. However it was detected fecal contamination in the beach water, for which the main origin was determined to be, in previous studies, the seagull feces.

The aim of this study was to analyze the antibiotic resistance of Escherichia coli isolates from the Berlengas beach water, gull feces and from the only human-derived wastewater effluent in the Island and so to assess the risk to public health of the fecal contamination and confirm its origin. It was also a goal to identify markers based on antibiotic resistance to fecal pollution sources discrimination.

In this sense we proceeded to the classification of the 414 Escherichia coli isolates from different sources in accordance with the main phylogenetic groups (A, B1, B2 and D), and over 70% of the isolates belonged to groups A and B1, usually associated with commensal strains. These two groups, along with group D, were the most frequent in all sources. Group B2 was only present in effluent (10.1%) and in a lower percentage in feces (3.9%).

The assessment of antibiotic susceptibility was performed for all isolates using the disk diffusion method. Overall, high percentages of resistance to the antibiotics tested were detected in the Berlengas island, particularly to penicillins, aminoglycosides and tetracyclines. Resistance to 3rd generation cephalosporins, to imipenem and ciprofloxacin was rare but more frequent in effluent isolates. It was also observed a global high rate of multiresistant isolates (around 30%). It was investigated the genetic basis for the phenotypes obtained, and bla_{TEM}, tet(A) and sul2 genes were the most frequently detected genes. $bla_{CTX\text{-}M\text{-}1}$ was detected in one water isolate and $bla_{CMY\text{-}2}$ in one feces isolate. The genetic context determined for these two genes was identical to what has been described for clinical isolates.

There were significant differences between the resistance patterns of water and the effluent. Some phenotypes and genotypes observed in water were only present in one of the other two sources: feces (in major number) and effluent. The potential use of these phenotypes and genotypes as markers of these pollution sources must be further investigated.

This study demonstrates that fecal pollution associated to gull feces, though generally considered less dangerous than human fecal pollution, may also constitute a risk to public health.

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INDEX OF ABREVIATIONS

µL: micro litter

6-APA: 6-amonipenicillanic acid

AMC: Amoxicillin/ clavulanic acid

AML: Amoxicillin

AMP - Ampicillin

AR: Antibiotic Resistance

ARA: Antibiotic Resistance Analysis

ARGs: Antibiotic Resistance Genes

BOX-PCR: BOX elements – polymerase chain reaction

bp: base pare

C: Chloramphenicol

CAZ: Ceftazidime

CIP: Ciprofloxacin

CLSI: Clinical and Laboratory Standards Institute

CN: Gentamicin

CTX: Cefotaxime

d-Ala–d-Ala: d-alanyl–d-alanine

dH_2O: distilled water

DHFR: dihydrofolate reductase

DHPS: dihydropteroate synthase

DNA: deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

ESLBs: Extended-Spectrum β-lactamase(s)

g:grams

HGT: Horizontal Gene Transfer

IMP: Imipenem

KF: Cefalotin

LA: Luria Bertani Agar

LB: Luria Bertani Broth

M: Molar

MGEs: Mobile Genetic Elements

min: minutes

mL: milliliter

MLS: Macrolides, Lincosamides, Streptogramins

MST: Microbial Source Tracking

NA: Nalidixic acid

no: number

ºC: degrees Celsius

PBP: Protein Binding Protein

PCA: Principal Component Analysis

PCR: polymerase chain reaction

pmol: picomole

PRL: Piperacillin

RDA: Redundancy Analysis

S: Streptomycin

SXT: Sulfonamide+trimethoprim

TAE: Tris-Acetato EDTA

TE: Tetracyclin

TE: Tris-EDTA

TZP: Piperacillin/ tazobactam

UNESCO: United Nations Educational, Scientific and Cultural Organization

UV: Ultraviolet

VRE: Vancomycin-resistant enterococci

I. INTRODUCTION

1. Antibiotics

In 1929 Alexander Fleming discovered that the fungi Penicillium notatum produced penicillin, a compound with antibacterial properties (Fleming 1929). In 1942 Waksman denominated "antibiotics" all the natural compounds produced by microorganisms that inhibit microbial growth or have microbiocide effect (Sousa 2005).

Natural antimicrobials are products of the microorganisms' secondary metabolism differing from the synthetic ones, or semi-synthetics, like the ones obtained through the core of penicillin 6-amonipenicillanic acid (6-APA), like ampicillin. With the increasing number of synthetic compounds with antibacterial activity, nowadays the term antibiotic denominates all the natural and synthetic antibiotics (Sousa 2005).

Antibiotics are used in human and veterinary medicine to treat and prevent diseases, and are also widely used in agricultural practices. The use of antibiotics to treat infectious diseases had a great impact on human morbidity and mortality rates raising life expectancy (van Hoek, Mevius et al. 2011). Ideally antibiotics should be highly efficient in the elimination of the infectious agent while having low or no toxic effects on eukaryotic cells. So, each group of antibiotics has a target that is specific to prokaryotic cells, thus reducing their negative effects in animals when taken in therapeutic doses (Phillips, Casewell et al. 2004; Sousa 2005; van Hoek, Mevius et al. 2011).

1.1. Classes of antibiotics and their mechanisms of action

Nowadays numerous different classes of antimicrobial agents are known and they are classified based on their mechanisms of action and on their spectrum of activity. For example, broad spectrum antibiotics can be effective against Gram-positive and Gram-negative bacteria (Neu 1992; van Hoek, Mevius et al. 2011). The different classes of antibiotics are listed on tables I.1. (β-lactams) and I.2 (other classes); β-lactams are presented separately due to its relevance and its several sub-classes.

Table I.1 –The class of β-lactam antibiotics and its sub-classes. Between parentheses the year of the discovery of the first antibiotic (Sousa 2005; van Hoek, Mevius et al. 2011).

Classes			Antibiotics
β-lactams (1929)	**Penicillins**		**Penicillin G** Ampicillin Amoxicillin Ticarcillin Piperacillin
	Cephalosporins	1º G.	Cefaloridin Cefalotin
		2º G.	Cefaclor Cefamandol
		3º G.	Ceftazidime Cefotaxime
		4º G.	Cefepime
	Monobactams		Aztreonam
	Carbapenems		Ertapenem Imipenem
	β-lactam inhibitors		Tazobactam Clavulanic Acid

1.1.1. β-lactams

The β-lactams play a major role in therapeutics due to their low toxicity and high efficiency (van Hoek, Mevius et al. 2011).

These antibiotics inhibit cell wall synthesis, as summarized in table I.3., by binding to the so-called penicillin-binding proteins (PBPs) and by interfering with the structural cross linking of peptidoglycans preventing terminal transpeptidation in the bacterial cell wall. As a consequence the cell wall is weakened and the final result is cytolysis or death due to osmotic pressure (Kotra and Mobashery 1998; van Hoek, Mevius et al. 2011).

The β-lactam family of compounds is characterized by a β-lactam nucleus in the molecular structure and includes the sub- classes listed in Table I.1. A short description of those that were used in the present work is given below:

Penicillins and derivatives: reduced spectrum of activity and susceptible to hydrolysis by the bacterial enzymes β-lactamases. The development of new molecules derived from 6-APA made possible to obtain semi-synthetic penicillins, like ampicillin, amoxicillin and piperacillin (Sousa 2005).

Cephalosporins: Semi-synthetic antibiotics grouped in first, second, third, and forth generation cephalosporins according to the timing of introduction in in therapeutics and to their spectrum of activity. In what concerns the spectrum of activity, fourth generation cephalosporins, display a broader spectrum of activity (Sousa 2005).

Carbapenems: are broad spectrum antibiotics, the most active ones belong t the β-lactam family (Sousa 2005).

β-lactam inhibitors: These compounds are usually combined with other β-lactams to combat strains that express β-lactamases (van Hoek, Mevius et al. 2011).

1.1.2. Other classes of antibiotics

Besides β-lactams, there are other antibiotic families like sulfonamides (with or without trimethoprim), macrolides, lincosamides, streptogramins, (the MLS group), fluoroquinolones, tetracyclines, aminoglycosides and glycopeptides, listed in table I.2.

Table I.2 - Antibiotics and their classes (except β-lactams which are listed in table I.1.). Between parentheses the year of the discovery of the first antibiotic of each class (in bold) (Sousa 2005; van Hoek, Mevius et al. 2011).

Classes		Antibiotics
Sulfonamides (1932)		Sulfadiazine Sulfamethoxazole + Trimethoprim
Aminoglycosides (1940)		**Streptomycin** Kanamycin Neomycin Gentamycin
Phenicols (1947)		Chloramphenicol
Tetracyclines (1948)		**Chlortetracycline** Minocycline Tetracycline
Glycopeptides (1950)		**Vancomycin** Teicoplanin
MLS (1950)	**Macrolides**	**Erythromycin** Clarithromycin Azithromycin
	Lincosamide	Clindamicin Lincomicin
	Streptogramin	
Quinolones (1962)	**Nalidixic Acid** Ciprofloxacin	

In table I.3. are listed the mechanisms of action of the antibiotics of each of those classes.

The **inhibition of protein synthesis** is achieved thanks to the affinity of antibiotics such as aminoglycosides (Vakulenko and Mobashery 2003), chloramphenicol (Schwarz, Kehrenberg et al. 2004), MLS and tetracycline, for the peptidyltransferase of the 50S ribosomal subunit of 70S ribosomes. Aminoglycosides interfere also with bacterial cell membranes inhibiting the translation of key proteins (Davies and Wright 1997; van Hoek, Mevius et al. 2011).

Quinolones inhibit n**ucleic acids synthesis** by inactivating DNA gyrase and topoisomerase IV (Hooper 2000; van Hoek, Mevius et al. 2011).

Bacterial cell wall has a terminus of d-alanyl–d-alanine (d-Ala–d-Ala) to which glycopeptides bound inhibiting the transglycosylation reaction and consequently the cell wall synthesis (Gao 2002; van Hoek, Mevius et al. 2011).

Sulfonamides and trimethoprim inhibit the bacterial growth by **modifying the energy metabolism** of the microbial cell. These two compounds competitively bind to two enzymes of the folate biosynthetic pathway, dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) respectively, inhibiting the enzymes activity: the products of the enzymes activity, such as thymine for bacterial cell growth and folic acid, aren't produced. Since 1968, the combination of trimethoprim and sulfamethoxazole has been used extensively because at certain concentrations it results in a synergetic bactericidal effect and reduces selection of antibiotic resistance (AR) consequently reducing associated costs (Roberts 2002; van Hoek, Mevius et al. 2011).

Table I.3. - Mechanisms of action of the most relevant antibiotic classes (Sousa 2005; van Hoek, Mevius et al. 2011).

Class	Mechanism of action
β-lactams	Inhibit the cell wall synthesis
Sulfonamides	Alter the energy metabolism
Trimethoprim	Alter the energy metabolism
Aminoglycosides	Inhibit protein biosynthesis and/or alter the bacterial CM
Phenicols	Inhibit protein synthesis
Tetracyclines	Inhibit protein synthesis
Glycopeptides	Inhibition of the cell wall synthesis
MLS	Inhibit protein synthesis
Quinolones	Inhibition of synthesis or metabolism of nucleic acids

2. Antibiotic resistance

The introduction of penicillin in the clinics altered the infections therapeutics reducing morbidity and mortality. However a rapid emergence of resistance in Staphylococcus aureus due to a plasmid-encoded penicillinase was soon observed (Jevons, Coe et al. 1963). The production of this β-lactamase was then detected in clinical isolates of several Staphylococcus species (Bradford 2001; van Hoek, Mevius et al. 2011).

In fact, AR was identified before the release of the first antibiotic for therapeutics: the first β-lactamase was identified in Escherichia coli prior to the release of penicillin for use in medical practice (see Table I.4. in bold) (Abraham and Chain 1940).

Tabela I.4. - Antibiotic discovery and resistance development (Products 1999).

Antibiotic	Introduction into clinics	Resistance identified
Penicillin	**1943**	**1940**
Streptomycin	1947	1947
Tetracycline	1952	1956
Erythromycin	1955	1956
Vancomycin	1972	1987
Nalidixic acid	1962	1966
Gentamicin	1967	1970
Fluoroquinolones	1982	1985

The aminoglycoside class was, along with the β-lactams, one of the first groups of antibiotics to which resistance emerged (see table I.4.): initially, the resistance that emerged in organisms such as Mycobacterium tuberculosis was restricted to modification of the antibiotic targets, however, enzymatic inactivation of the antibiotic has emerged as an important mechanism in both Gram-positive and Gram-negative bacteria (Davies and Wright 1997; Wright 1999).

Over the years it has been shown by numerous studies that antibiotic use/consumption contributes to the devellopment of AR in bacteria (Goossens, Ferech

et al. 2005; Hulscher, Grol et al. 2010; van Hoek, Mevius et al. 2011). Vancomycin-resistant enterococci (VRE), isolated in 1988 constitute an example of the link between antibiotic usage and resistance development: dissemination of the resistance gene to vancomycin in humans happened not just because of the large use of the antibiotics in the clinical practice but also because glycopeptide avoparcin (an analogue of vancomycin) was used in animal food as a growth promoter. In 2006 the use of all antibiotics as growth promoters in animals was forbidden in the European Union (Commission 2008) for the reason that employing antibiotics as growth promoters largely contributes to the emergence of AR (Sousa 2005; van Hoek, Mevius et al. 2011).

The presented examples show that microbes reacted to the changed environment (selective pressures imposed by the use of antibiotics) adapting by developing resistance to antibiotics using a variety of mechanisms. Moreover, their ability of interchanging genes by horizontal gene transfer (HGT) has an important role in the evolution and dissemination of resistance as it will be described next.

2.1. Intrinsic and acquired resistance

An example of intrinsic resistance is the resistance displayed by antibiotic producing strains that already have a natural resistance to that specific antibiotic being a part of their genetic inheritance. In other microorganisms intrinsic resistance mechanisms encoded in their resistome - the set of antibiotic resistance genes (ARGs) - and expressed at a basal level, confer to them a naturally reduced susceptibility to the drugs (Sousa 2005; van Hoek, Mevius et al. 2011; Lupo, Coyne et al. 2012).

In contrast to intrinsic resistance, acquired AR is not part of the natural characteristics of a species/genus but appears as a result of genetic information transfer induced by selective pressure in some strains of a species/genus. One of the main selective forces has been the human use of antibiotics (Martinez 2009).

HGT events play the major role in the rapid emergence of AR among bacteria being responsible for the transfer of resistance genetic determinants from antibiotic producers or species provided with intrinsic AR mechanisms to commensal and pathogenic bacteria. Besides clinics, other activities where the use of antibiotics is intensive contribute to the emergence and dissemination of AR, namely veterinary, aquaculture, and agriculture (EARS-Net 2011; Lupo, Coyne et al. 2012).

2.1.1. Mechanisms of antibiotic resistance transfer

Antibiotic resistance genes (ARGs) are frequently located in mobile DNA elements. These elements share the ability to move within a genome and/or to other bacterial cells. DNA transfer is usually mediated by mobile genetic elements such as plasmids, transposons and genomic islands or by bacteriophages. However free naked DNA can also be captured and incorporated into the genome in a process designated transformation.

If a resistance gene is on a conjugative (contain all the genetic information required to transfer from one bacterium to another) or mobilizable plasmid (use the conjugation functions of co-resident conjugative elements to transfer to another host) then it has the potential to be transferred to new hosts. Broad host range plasmids can transfer to several phylogenetically distant species and are particularly effective in the dissemination of AR (van Hoek, Mevius et al. 2011; Lupo, Coyne et al. 2012).

Integrons are not mobile elements sensus stricto but genetic platforms that are responsible for integration and rearrangements of resistance determinants called gene cassettes (Koczura, Mokracka et al. 2012). Genes involved in resistance to almost all antibiotic families have been found in integrons, including β-lactams, aminoglycosides, trimethoprim, chloramphenicol, fosfomycin, macrolides, lincosamides, rifampicin, and quinolones (Stalder, Barraud et al. 2012). Integrons linked to mobile DNA spread horizontally through bacterial population and so disseminates all the resistance genes contained in their gene cassettes (Stalder, Barraud et al. 2012).

Based on the amino acid sequence of the IntI protein, five classes of AR integrons have been described (Cambray, Guerout et al. 2010). Classes 1, 2, and 3 are the most commonly detected. For instance it has been reported that multidrug resistance among Enterobacteriaceae is associated with the presence of class 1 integrons (Leverstein-van Hall, Blok et al. 2003). Class 1 integrons have been extensively studied due to their broad distribution among Gram-negative bacteria of clinical interest and are the most reported in bacteria associated to humans or other animals (Stalder, Barraud et al. 2012).

It's easy to conclude that, given all the mentioned mechanisms, bacterial cells have a large potential to adapt by acquiring genes of resistance to antibiotics from other cells or from the environment. This makes the dissemination of resistance to antimicrobial agents an emergent problem with some microorganisms becoming extremely resistant to existing antibiotics. AR and virulence can, in extreme cases, be shared by the same strain raising a public health concern: an example was the strain that caused a large epidemic outbreak of E. coli O104:H4 in Europe (Brzuszkiewicz, Thurmer et al. 2011; Wu, Hsueh et al. 2011).

2.2. Mechanisms of resistance

Bacteria have become resistant to antimicrobials through a number of mechanisms (see Table I.5.).

2.2.1. β –lactams

The production of β-lactamases is the most effective and widely disseminated mechanism of resistance to β-lactams. These enzymes inactivate the antibiotics by hydrolyzing the β-lactam ring present in their chemical structure. More than 900 different β-lactamases (Jacoby and Bush), encoded by bla resistance genes, have been identified and they differ in their molecular characteristics and in the number of different β-lactams that they hydrolyze (spectrum of activity) (van Hoek, Mevius et al. 2011).

Ambler classes (the four classes A, B, C and D) (Ambler 1980) classify β-lactamases according to their molecular characteristics: β-lactamases from classes A, C and D have serine at their active site; class B β-lactamases are metallo-enzymes that require zinc for their catalytic activities (Li, Mehrotra et al. 2007; Bush and Jacoby 2010; van Hoek, Mevius et al. 2011).

β-lactamases have different spectrum of activity: broad spectrum β –lactamases provide resistance to penicillins and older cephalosporins; extended-spectrum β-lactamases (ESBLs) confer resistance to penicillins, first-, second-, and third-generation cephalosporins and monobactams, but not to carbapenems and are inhibited by β-lactamase inhibitors (van Hoek, Mevius et al. 2011).

Among the class A β-lactamases, the ESBLs represent a public health concern. The main families are TEM, SHV, CTX-M, VEB, and GES enzymes. Among them, the highest number of variants described in the last years corresponds to the CTX-M family (Canton, Gonzalez-Alba et al. 2012).

The mechanisms of β-lactam resistance include, besides deactivation by β-lactamases, inaccessibility of the antibiotics to their target enzymes, modifications of target enzymes and efflux bombs (Li, Mehrotra et al. 2007; Bonnedahl, Drobni et al. 2009).

Table I.5. – Examples of acquired ARG and mechanisms (Sousa 2005; van Hoek, Mevius et al. 2011).

Antibiotics	Resistance Genes	Resistance mechanism
β-lactams	oxa, ges, imp, vim, tem, shv, ctx-m,	Enzymatic hydrolysis by β-lactamases
Sulfonamides	dfr sul	Mutation on DHFR (drf) and DHPS (sul) genes
Aminoglycosides	aac aad aph, ant amr, rmt, npm str sat npt	Enzymatic modification by: aminoglycoside acetiltransferases, adenylyltransferases, phosphoryltransferases; methyltransferases streptothricin acetyltransferase streptothricin acetyltransferase neomycin phosphotransferase
Phenicols	cat cml cmr	Chloramphenicol acetyltransferase efflux
Tetracyclines	tet(A), (B), (C),(D),(E) tet(M), (O),(S), (K),(W) otr	Efflux proteins Ribosomal protection proteins Oxytetracycline resistance
Glycopeptides	vanA, B,C, D,E,G	Modified peptidoglycan precursors
MLS	erm mph, ere, inu, vat mef, msr	rRNA methylase Inactivating enzyme Efflux
Quinolones	qnr aac(6')-Ib-cr qepA	Protector proteins Aminoglycoside acetyltransferase Efflux

2.2.2. Sulfonamides and Trimethoprim

Resistances to sulfonamides and trimethoprim are encoded by mutations located on highly conserved areas of DHPS genes (sul) and DHFR genes (dfr) related with the antibiotic mechanism of action. Sulfonamide resistance is conferred by changes in the sul genes. The most widespread trimethoprim resistance mechanism is the replacement of a trimethoprim-sensitive DHFR by a plasmid-, transposon-, or cassette-borne trimethoprim-resistant DHFR (Skold 2001; Sousa 2005; Zhang, Zhang et al. 2009).

2.2.3. Aminoglycoside

There are several different biochemical mechanisms of resistance to aminoglycoside antibiotics: reduced uptake, mutational modification of 16S rRNA and of ribosomal proteins, enzymatic modification of 16S rRNA through rRNA methylases and enzymatic modification of the antibiotic - the most commonly identified aminoglycoside resistance mechanism (Davies and Wright 1997; van Hoek, Mevius et al. 2011).

2.2.4. Chloramphenicol

The identified chloramphenicol resistance systems are based on inactivating enzymes like acetyltransferases – CATs encoded by genes catA and catB – and phosphotransferases, mutations of the target site, permeability barriers, and efflux systems coded by the genes floR and cmlA (Schwarz, Kehrenberg et al. 2004; van Hoek, Mevius et al. 2011).

2.2.5. Tetracyclines

Tetracycline resistance mechanisms can be mediated by efflux pumps, ribosomal protection proteins or inactivating enzymes. The tet(A), (B), and (M) are among the most frequent tetracycline resistance genes and encode for efflux pumps (the first two) and ribosomal protection protein (the last) (van Hoek, Mevius et al. 2011).

2.2.6. Glycopeptides

It was mentioned before that glycopeptides bound to the bacterial cell wall terminus of d-Ala–d-Ala inhibiting the transglycosylation reaction and consequently the cell wall synthesis. By glycopeptides' target modification the binding affinity decreases and the cell wall synthesis may continue normally. For example the van genes encode for modified peptidoglycan precursors and their expression results in vancomycin resistance. The vanA and vanB genes have been found associated to plasmids resulting in their widespread dissemination. The other reported van genes are chromosome encoded (van Hoek, Mevius et al. 2011).

2.2.7. MLS

Commonly, MLS resistance results from the expression of erm genes that encode rRNA methylases. The activity of these enzymes prevents the binding of the antibiotic to the ribosome. Although structurally unrelated, the same resistance mechanism (expression of erm genes) confers resistance to macrolides, lincosamide, and streptogramin (Roberts 2002; Zhang, Zhang et al. 2009; van Hoek, Mevius et al. 2011).The erm genes are usually acquired and associated with mobile elements being easily transferred from one host to another (Roberts 2008; Zhang, Zhang et al. 2009).

2.2.8. Quinolones

As described in figure I.1. quinolone resistance mechanisms can be chromosome encoded or plasmid mediated. Chromosome encoded resistance by porin loss results in decreased outer membrane permeability; another intrinsic mechanism is activated in the presence of the antibiotic by the overexpression of naturally occurring efflux pumps genes; also contributing to resistance are mutations of the molecular targets DNA gyrase genes (gyrA and B) and topoisomerase IV genes (parC and E) in the quinolone resistance determining regions. In concern to plasmid-mediated resistance, qnr genes encode proteins that protect DNA gyrase and topoisomerase IV from quinolone inhibition; aac(6)-Ib-cr gene encodes an aminoglycoside acetyltransferase, which has two amino acid changes (Trp102Arg and Asp179Tyr) conferring to the enzyme the ability to acetylate ciprofloxacin. The qepA gene encodes a plasmid-mediated efflux pump which can extrude hydrophilic fluoroquinolones like ciprofloxacin (Jacoby 2005; Yamane, Wachino et al. 2007; Strahilevitz, Jacoby et al. 2009).

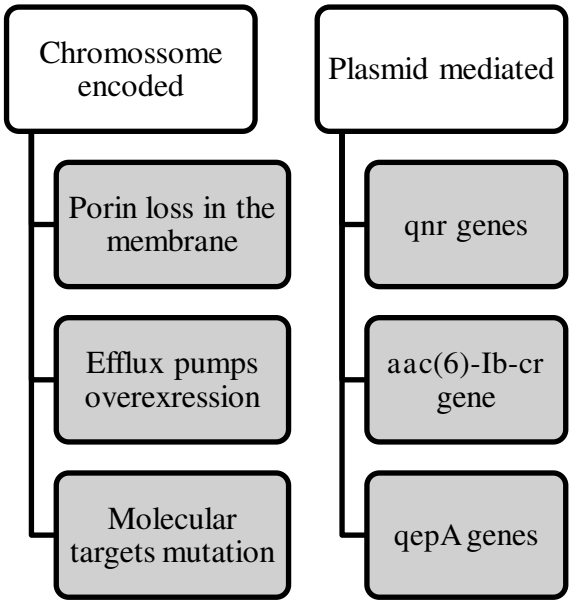


Figure I.1. - Mechanisms of resistance to quinolones.

3. Environmental spread of antibiotic-resistant bacteria

Selective pressure for ARGs exists in nature leading to their naturally occurrence in a balance with the environment. Humans have applied additional selective pressures for ARGs because of the large quantities of antibiotics used in medicine and agriculture.

The incompletely metabolized antibiotics are the primary source of antibiotics in natural environments: depending on the type of antibiotic, between 30% and 90% of an administered dose given to humans and animals are excreted in the urine or feces as active substances and introduced to the sewage system being only partially eliminated in sewage treatment plants and ending up in the environment, reaching surface waters, groundwater, soils and sediments (Matyar, Dincer et al. 2004; Schlusener and Bester 2006; Kummerer 2009; Matyar, Akkan et al. 2010; Mudryk, Perlinski et al. 2010).

Baquero and Martínez (Baquero, Martinez et al. 2008) explained how antibiotics resulting from the human activity get into the environment and how ARGs disseminate dividing the process in compartments, called reactors, each one favorable for genetic evolution. According to these authors there are four main genetic reactors in which AR evolves (Figure I.2.):

- The primary reactor is constituted by the human and animal microbiota in which antibiotics exert their actions;
- The secondary reactor involves places and facilities (like hospitals and farms) in which susceptible individuals interact and are exposed to bacterial exchange;
- The tertiary reactor corresponds to the wastewater and other biological residues originated in the secondary reactor in which bacterial organisms from many different individuals have the opportunity to mix and exchange genes;
- The fourth reactor is the soil and the water environments, where the bacteria from the previous reactors mix and counteract with environmental organisms.

Particularly in the lowest reactors, bacteria from human or animal-associated microbiota (in black in figure 2) mix with environmental bacteria (in white in figure 2), leading to genetic variation and to the emergence of new resistance mechanisms that are re-introduced in human or animal environments (back arrows in figure 2) (Baquero, Martinez et al. 2008; Martinez 2009).

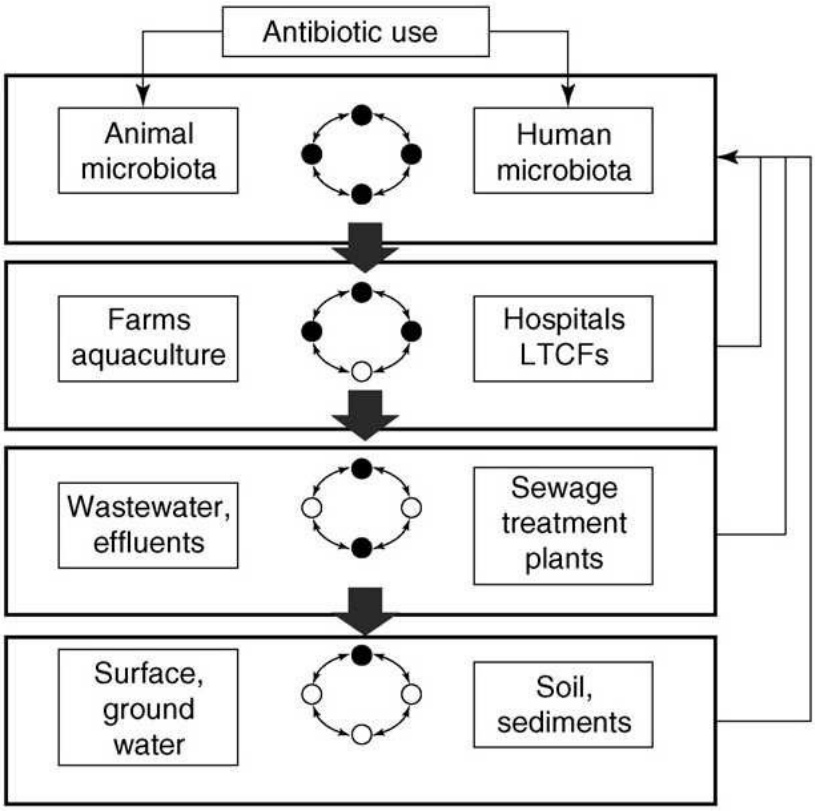


Figure I.2. - The four main genetic reactors (Baquero, Martinez et al. 2008).

3.1. The role of HGT in the spread of ARG in the environment

As referred before HGT events play the major role in the rapid emergence of AR and it has been long recognized that various stress conditions may contribute to increased rates of HGT. For example, UV irradiation or starvation affects the mobility of transposons and insertion sequences as well as the sub-inhibitory concentrations of antibiotics may significantly increase the frequency of horizontal transfer of many types of MGEs (Aminov 2011). In the environment microorganisms are exposed to the mentioned stress conditions so HGT may happen at increased rates (Martinez 2009; Allen, Donato et al. 2010).

In Thimm et al experiment in 2001 in soil microcosms, with E. coli as a donor of a genetically marked large conjugative plasmid RP4luc, in the presence or absence of earthworms was observed that MGEs from soil have entered the earthworm gut and that the plasmid was transferred at higher frequencies than estimated in laboratory. These results confirm the earlier notion that microbial ecosystems are not isolated and there is a potential for lateral gene exchange among different microbial ecosystems. If MGEs from soil have entered the earthworm gut, then they can also enter the gut of animals that are next in the food chain, for example, moles and birds (Thimm, Hoffmann et al. 2001).

In conclusion, there is an enormous diversity of ARGs in the environmental microbiota, that accumulated during billions of years of evolution and there are no barriers among the ecological compartments in the microbial world. The gene pools of microbiota from different compartments maintain a permanent flux mediated by the different mechanisms of HGT and so resistance genes reach easily pathogenic microorganisms (Aminov 2011).

3.2. Aquatic ecosystems as reservoirs of antibiotic resistance genes

Water is an important vector of AR dissemination. It is involved as a crucial agent in all four genetic reactors mentioned above (Figure I.2.; Martinez 2009), but particularly in the last ones, receiving bacteria from different sources (wastewater treatment plants, water from urban effluents or used in industrial and agriculture activities) that will react with the indigenous bacteria. This mixing makes possible the exchange of ARGs between the indigenous bacteria with intrinsic resistance mechanisms and the animal and human bacteria already selected for AR. Disinfectants and heavy metals are also released in water and beyond the ecological damage that these compounds induce in water communities, they also exert a selective pressure that results in a selection for AR microorganisms (Aminov and Mackie 2007; Baquero, Martinez et al. 2008).

So, water plays a key role as support medium for acceptors and donors of ARGs that subsequently may spread among bacteria from different ecosystems. This exchange has been proven in many studies like the investigation of Cernat et al in 2007 on the occurrence and distribution of various resistance genes in multiple antibiotic resistant E. coli strains isolated from various aquatic sources and also their transmission pattern. It

was observed that all strains showed a wide variety of ARGs, some of them in class 1 integrons (Cernat, Balotescu et al. 2007). Also Agerso and Petersen have found that tet(E) gene is often located on large horizontally transferable plasmids of Aeromonas spp. isolated from pond water of a fish farm, capable of interspecies transfer to E. coli. (Agerso and Petersen 2007).

In figure I.3. it is possible to observe the geographical distribution of ARGs: studies detected these resistance genes to different antibiotic classes all around the world in aquatic environments. In Europe, nearly all types of ARGs were detected in aquatic environments of some countries including Portugal, for example, in bacteria from a slaughterhouse waste water treatment plant (Moura, Henriques et al. 2007; Zhang, Zhang et al. 2009). Therefore water may act as a reservoir of ARGs and as a vector of resistant organisms.

Wastewaters greatly contribute to this scenario. Bacteria carrying ARGs (and perhaps naked DNA) in wastewaters of hospital, animal production, and fishery areas can be transported to the nearby streams, rivers, lakes, or other aquatic bodies or enter through the soil during rainfall. ARGs frequently detected in these types of wastewaters include for instance chloramphenicol resistance genes (cat1, 4 and B3) in the aquaculture systems and sulfonamide resistance genes (sul1, 2, 3 and A) in fish farms (Dang, Zhang et al. 2006; Agerso and Petersen 2007; Zhang, Zhang et al. 2009).

Untreated sewage was found to contain a variety of ARGs encoding resistances to all classes of antibiotics. The environmental conditions of activated sludge and the biofilms facilitate horizontal transfer of the ARGs from one host to another because of the nutritional richness and high bacterial density and diversity (Schluter, Szczepanowski et al. 2007; Zhang, Zhang et al. 2009).

Surface water and shallow groundwater are commonly used as source of drinking water; thus, ARGs can go through drinking water treatment facilities, enter into water distribution systems and into human food chain (Figueira, Serra et al. 2012; Vaz-Moreira, Figueira et al. 2012; Vaz-Moreira, Nunes et al. 2012). Humans get in contact with water and consequently with ARGs not only through the food chain but also in recreation activities leading to the introduction of new resistance mechanisms in clinic (Schwartz, Kohnen et al. 2003; Walsh, Ingenfeld et al. 2011).

Figure I.3. - Detection of the antibiotic resistance genes in geographically isolated water environments. Genes encoding resistance to aminoglycosides (red square), chloramphenicol (brown inverted triangle), β-lactam (plus symbol), macrolide (sky blue triangle), sulfonamide (violet diamond), tetracycline (green circle) and trimethoprim (indigo star) (Zhang, Zhang et al. 2009).

3.3. Public health concern: ARGs as environmental pollutants

If AR further depletes the number of effective drugs available, therapeutic options will be restricted, which represents a serious public health problem resulting from the widespread dissemination of ARGs. For this reason and also because ARGs are widely distributed in various environmental compartments having also an impact in the equilibrium of the ecosystems, these genes have been considered environmental emerging pollutants (Gottlieb and Nimmo 2011).

The results of the recent surveillance report of antimicrobial resistance in Europe show a general Europe-wide increase of antimicrobial resistance in the gram-negative pathogens under surveillance (E. coli, Klebsiella pneumoniae and Pseudomonas aeruginosa), whereas the occurrence of resistance in the gram-positive pathogens (Streptococcus pneumoniae, Staphylococcus aureus, Enterococcus faecium and Enterococcus faecalis) appears to be stabilizing or even decreasing in some countries (EARS-Net 2011).

A north-to-south gradient is evident in Europe: in general, lower resistance percentages are reported in the north and higher percentages in the south of Europe. This may reflect differences in infection control practices and antibiotics use in the reporting countries. Prudent use of antibiotics and infection control measures should be cornerstones of prevention and control efforts to reduce the selection and transmission of AR bacteria. (EARS-Net 2011)

The fact that ARGs can spread and exchange among environmental microorganisms of different genera and cohabitationwith organisms of completely different kingdoms and the appearance of potential antibiotic resistances in drinking water distribution systems of some nations or regions requires increased surveillance for risk assessment and prevention strategies to protect public health (Agerso and Petersen 2007; Zhang, Zhang et al. 2009; Figueira, Vaz-Moreira et al. 2011; Vaz-Moreira, Nunes et al. 2011; Vaz-Moreira, Figueira et al. 2012).

It is necessary the implementation of measures to improve water resources like establishing the AR detection as a water quality parameter and developing efficient methods to this detection (Lupo, Coyne et al. 2012).

4. Antibiotic-resistant bacteria in coastal environments

In various bacterial species in sediments of Tokyo Bay, Sagami Bay, and the open Pacific Ocean was found the tetracycline resistance gene tet(M) (Rahman, Nonaka et al. 2008). The numbers of oxytetracycline-resistant bacteria increased in sediments around a marine aquaculture site after oxytetracycline therapy, and tet(M) was evident in both Gram-positive and Gram-negative bacteria from various genera in the sediments of the marine environment (Neela, Nonaka et al. 2007; Rahman, Nonaka et al. 2008). The marine sediments can be considered as natural reservoirs of ARGs, particularly tetracycline resistance. ARGs in sediments are selected due to antibiotic presence or they settle from water.,

Coastal waters, frequently used by humans for recreation, also constitute a natural reservoir of ARGs as it has been demonstrated in studies in these areas (Mudryk, Perlinski et al. 2010; Simoes, Poirel et al. 2010; Matyar 2012) and as it will be described next.

4.1. Antibiotic resistance genes in coastal waters

Beaches of marine coasts are dynamic environments subjected to natural changes in terms of physico-chemical variables, nutrients and to strong anthropogenic pressures from various activities. Bacteria inhabiting these ecosystems are well adapted to these versatile conditions. The previously reported studies suggested that human activity may play an important role as a source of AR in marine beaches (Mudryk, Perlinski et al. 2010; Alouache, Kada et al. 2012).

Results from description of AR in seawater on the beaches of Algeria, showed a significant level of resistance to antibiotics, particularly β-lactams, detected mainly among saprophytic environmental bacteria. Transmissible ESBLs of CTX-M-15 type were detected in E. coli meaning that resistance genes can disseminate in these environments representing an health risk from recreational water contact (Alouache, Kada et al. 2012).

Maravic et al found in coastal waters of the Adriatic Sea in Croatia Burkholderia cepacia showing multiple AR. Two of the isolates produced a chromosomal encoded ESBL (TEM-116) mostly found in members of the Enterobacteriaceae family indicating HGT (Maravic, Skocibusic et al. 2012).

The results from Dada et al study on the occurrence of antibiotic-resistant enterococci in coastal bathing waters in Malaysia suggested that samples from Port Dickson may contain multiple AR bacteria and that this could be due to high-risk fecal contamination from sewage discharge pipes that drain into the seawater (Dada, Ahmad et al. 2012).

4.2. Seagulls and antibiotic resistance dissemination

Migratory birds can acquire and spread resistance genes through geographically disperse environments because they travel long distances and inhabit many different places. Proximity to human activity increases the number of the antibiotic-resistant bacteria that are associated with wild birds. For instance, gulls carrying higher levels of antibiotic-resistant E. coli are the ones inhabiting near waste or agricultural water in contrast with the ones inhabiting human-unrelated sites (Dolejska, Bierosova et al. 2009; Literak, Dolejska et al. 2009).

Nevertheless, AR is also described in remote bird populations: in arctic birds, 8% of E. coli isolates were recently found to be resistant to at least 1 of 17 antibiotics tested, and 4 were resistant to 4 or more antibiotics. One isolate was resistant to cefadroxil, cefuroxime and cefpodoxime, a common pattern in clinical isolates (Sjolund, Bonnedahl et al. 2008). Many birds breed in the arctic and migrate to up to six continents. They probably acquire antibiotic-resistant bacteria from environments that are under human influence or from other birds that contact with those environments. This study shows the geographical distances that can be travelled by bacteria which genomes encode ARGs that are associated with human selective pressures and the potential of migratory birds as vectors for antibiotic dissemination (Sjolund, Bonnedahl et al. 2008; Allen, Donato et al. 2010).

Gull populations have increased worldwide due in large part to the availability of human-derived products that they use as food along coastal areas, in parallel with the growth of human populations. Several recent studies have detected clinically relevant ARGs in gull feces (Wallace, Cheasty et al. 1997; Dolejska, Cizek et al. 2007; Benskin, Wilson et al. 2009; Radhouani, Poeta et al. 2009; Bonnedahl, Drobni et al. 2010; Simoes, Poirel et al. 2010).

ESBL producing E. coli isolates, fluoroquinolones-resistant isolates and isolates carrying class 1 and 2 integrons have been found in many gull's species around the

world (Poeta, Radhouani et al. 2008; Bonnedahl, Drobni et al. 2009; Dolejska, Bierosova et al. 2009; Wallensten, Hernandez et al. 2011).

Simões et al identified in seagull feces from Porto beaches, Portugal, a variety of ESBL–producing E. coli isolates, with a high rate of cefotaxime resistance. Beaches may therefore present a risk to public health because of the potential pathogen-spreading capacity of migratory birds (Simoes, Poirel et al. 2010).

Using functional metagenomics to characterize the diversity of the gull resistome, Martiny et al detected ARGs that are usually found in human and pet's bacteria like class A and C β-lactamases. In this investigation a large number of novel genes were identified (thirty one undescribed ARGs) showing that gulls can also introduce new AR mechanisms in the human microflora (Martiny, Martiny et al. 2011).

Gulls do not naturally come into contact with antibiotics. These birds can be infected with resistant strains and potentially serve as their reservoirs, vectors and bioindicators in the environment reflecting the presence of such resistant strains in their sources of food and/or water. The results from the mentioned investigations imply that ARGs arising from the intense use of antibiotics spread to wild life that can disseminate them, especially the migratory species, like gulls.

5. Antibiotic resistance in E. coli

E. coli belongs to the family Enterobacteriaceae consisting in a facultative anaerobic, gram-negative and nonsporulating rod. It has relatively simple nutrition requirements. E. coli is the best known microorganism and an inhabitant of the intestinal tract of humans and other warm-blooded animals; thus it is a commensal bacteria, playing an important nutritional role in the intestinal tract by synthesizing vitamins (Madigan, Dunlap et al. 2009).

Some strains of E. coli are pathogenic, like enteropathogenic E. coli implicated in gastrointestinal infections and generalized fevers and E. coli O157:H7, an enterohaemorragic strain that is in the origin of foodborne diseases and, in a small percentage of cases, can be life-threatening (Madigan, Dunlap et al. 2009).

Due to its characteristics of not normally being pathogenic to humans and its presence at higher concentrations than the pathogens, E. coli is used as an indicator of fecal pollution, a microorganism used to predict the presence of pathogenic microorganisms. It has also been frequently used for Microbial source Tracking (MST) in which isolates from different sources and from the contaminated place are genotypic or phenotypic characterized for determining the origins of fecal contamination (Scott, Rose et al. 2002; Roslev and Bukh 2011).

Phylogenetic analyses showed that E. coli stains can be assigned to four main phylogenetic groups: A, B1, B2 and D. These groups differ in their ecological niches, life-story and characteristics like the exploitation of sugar sources, growth rate and antibiotic-resistance profiles (Carlos, Pires et al. 2010). The stains belonging to groups B2 and D contain more virulence factors and the extraintestinal pathogenic bacteria usually belong to these groups while the commensal stains belong to groups A and B1 (Clermont, Bonacorsi et al. 2000; Carlos, Pires et al. 2010).

AR in clinical isolates of E. coli was well illustrated by the 2011 report from the European Antimicrobial Surveillance Network (EARS-Net 2011): resistance to β-lactams is mostly due to production of β-lactamases and resistance to third-generation cephalosporins (Figure I.4.) is mostly conferred by ESBLs being nowadays the CTX-M-type the most common ESBL in E. coli. There is an emergence of carbapenem resistance in E. coli, mediated by metallo-β-lactamases (such as the VIM or IMP enzymes, or the emerging NDM enzyme) or serine-carbapenemases (such as the KPC

enzymes).. In general, this report showed in many countries an increase during 2008 and 2011 of combined resistance to at least three antimicrobial classes and of E. coli strains producing multiple β-lactamases, two facts that are a subject of concern in terms of public health.

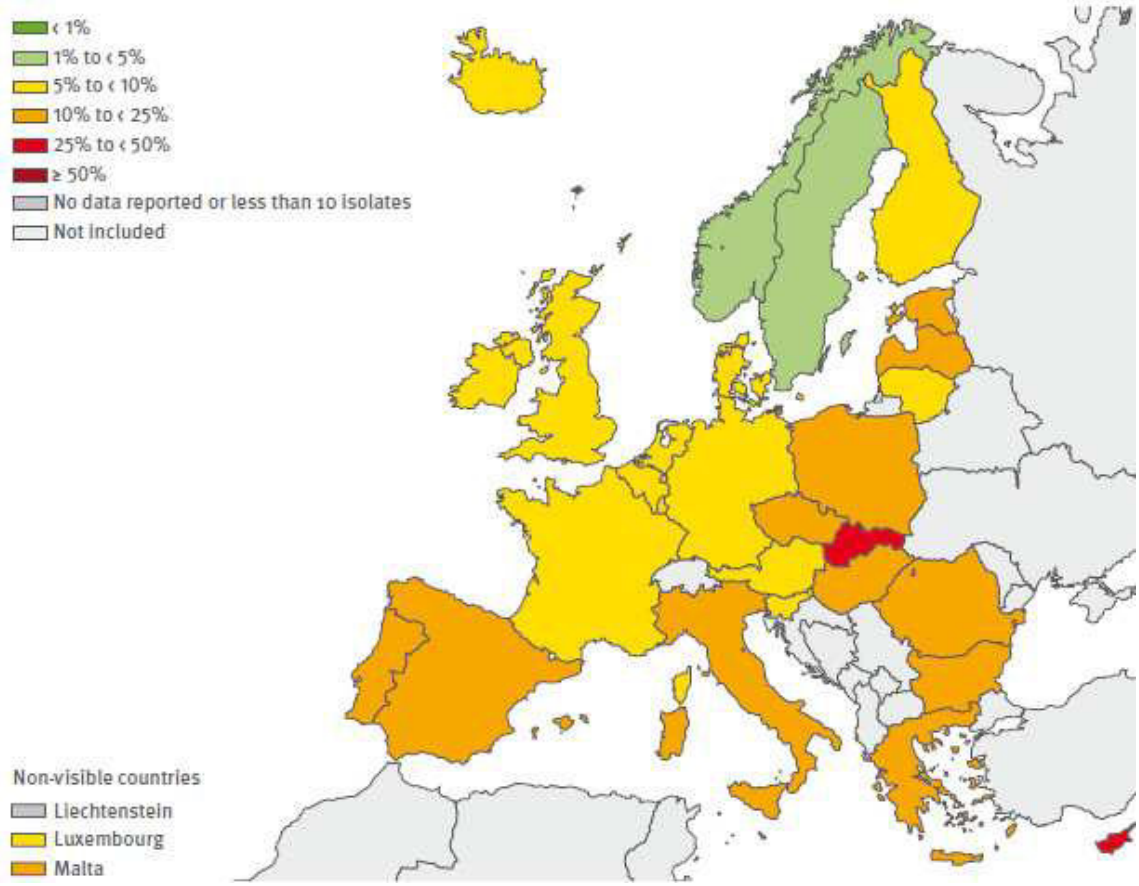


Figure I.4. - Percentage (%) of E. coli invasive isolates with resistance to third-generation cephalosporins by European Union country in 2011 (EARS-Net 2011).

Many studies detected antibiotic-resistant E. coli isolates in the environment including in aquatic environments: multiresistant E. coli have been reported in surface and river water worldwide (Hu, Shi et al. 2008; Figueira, Serra et al. 2011; Tacao, Correia et al. 2012) and even in drinking water in which multiple-antibiotic-resistant E. coli strains were found to carry ARGs encoding resistances to aminoglycoside, β-lactam, tetracycline, and trimethoprim-sulfamethoxazole (Cernat, Balotescu et al. 2007).

6. Berlengas as a model of coastal environment

Berlenga Island, located 5.7 miles from the Portuguese coast in Peniche, is classified by the Portuguese Government as Berlengas Natural Reserve since 1981, and was in 2011 added to the World Network of Biosphere Reserves by International Coordinating Council of UNESCO's Man and Biosphere Program (Queiroga, Leão et al. 2008).

The Berlengas archipelago is constituted by a granitic and metamorphic complex of rocks having a steep topography where is very common the formation of caves and underwater cracks. The location of Berlengas, in the transition zone between the European and Mediterranean sub regions is an important factor in the oceanographic dynamics of the region with an intense upwelling that allows the renewal of nutrients in the surface contributing to the great diversity of marine species and habitats (Queiroga, Leão et al. 2008; Araújo 2012).

Rich in avifauna, Berlenga Island is the shelter of one of the largest western colonies of the yellow-legged-gull (Larus cachinnans) and the nesting location of other seabirds like dark wing gull (Larus fuscus) and tridacttyl gull (Rissa tridactyla). It is also the point of concentration of migratory birds that feed in the surrounding sea (Queiroga, Leão et al. 2008; Araújo 2012).

The high number of visitors in the summer period can be a problem to the conservation of the archipelago ecosystem: that number often exceeds the limit of 350 visitors a day established by law (270/90 of April 10),pressuring the basic infrastructures of the island including water supply, sanitation and waste production (Queiroga, Leão et al. 2008).

Salt water is used for washing and to ensure the functioning of the rudimentary sanitary facilities in the houses of the fishermen's village and restaurant: seawater is pumped into tanks and used in toilets returning to the sea through ducts. Part of the water goes through a waste milling system and the washing water from the catering services is also released directly into the sea through the same pipeline system. There are no pits or other basic sanitation systems (Araújo 2012).

In the last few years the population of seagulls increased significantly. In 1995 there were 32.000 birds when various management measures were implemented consisting in the eradication of mature individuals and destruction of their postures.

However the population continued to increase spreading through the islands (Queiroga, Leão et al. 2008).

The recent results of the microbiological quality of the Berlenga beach water showed moments in which the maximum allowable value is exceeded (500 E. coli /100 ml) indicating fecal pollution (Araújo 2012).

6.1. MST in Berlenga Island

Once it is detected fecal contamination in a certain area it is of great importance to determine the origin of that contamination to understand the associated health risks and to know what to do to solve or minimize the pollution problem. MST aims to identify the origin of fecal pollution assuming that microbiota of the fecal pollution and the biological source have similar phenotypic or genotypic characteristics. The methods to evaluate and compare the microorganisms from the polluted area and the suspected sources can be culture- or library-dependent or independent. For instance, antibiotic resistance analysis (ARA) is a phenotypic, library and culture dependent MST method that differentiates bacteria from different sources through their AR profiles (Scott, Rose et al. 2002; Roslev and Bukh 2011).

In the last few years a problem of fecal contamination has been detected in the beach water of Berlenga Island. Seagulls were identified as the main source of fecal contamination in the scope of a previous study (Araújo S. 2013).

7. Aims of the work

AR dissemination is becoming a public health concern and a few studies in coastal environments concerning this subject show that beaches (sediments and seawater) also contain antibiotic-resistant bacteria constituting presumptive reservoirs of ARGs.

Berlenga Island is a protected area. Nevertheless, fecal contamination was detected in the Berlengas' seawater and seagulls were identified has the main origin of this contamination.

E. coli isolates from gulls and humans are subjected to different selective pressures. As a consequence it is expected that isolates display different levels of AR, also carrying different ARGs and MGEs, depending on the host.

We hypothesize that the levels of AR and the diversity of ARGs in water of the Berlenga beach will resemble the ones of the main origin of fecal contamination (e.g seagull feces).

Taking this into account, the major aims of this work are:

- to determine the AR levels in E. coli from seagull feces, human-derived sewage and water collected in the Berlenga beach.

– to characterize the genetic basis of the identified resistance phenotypes.

- to identify possible correlations between levels of resistance/diversity of ARGs andthe source of the isolates and E. coli phylogenetic groups.

- to identify AR-related markers (phenotypes and/or genotypes) with potential to discriminate the fecal pollution sources.

For these purposes the AR patterns of the E. coli isolates previously obtained from the different sources: beach water (n=166), seagull feces (n=179) and human-derived sewage (n=69) were determined. Based on the AR phenotypes, genes conferring resistance were investigated by PCR. The primers targeted ARGs usually found in MGEs and thought to represent acquired resistance, thus more likely to disseminate to humans' commensal microbiota, constituting a greater risk to public health.

This study will add data to better assess the risks to human health related to the contamination of coastal waters, using well delimited ecosystem of the Berlenga beach as a model. Also, it is expected that the obtained results will contribute to further clarify the role of seagulls as vehicles of dissemination of AR.

II. MATERIAL AND METHODS

1. Culture media

Two culture media were used in this study, Luria-Bertani Agar (LA) obtained by supplementing Luria-Bertani medium with Agar at 2% (m/V), and Mueller-Hinton agar, all acquired from Merck (Darmstadt, Germany). The culture media were autoclaved at 121ºC for 15 min. After cooled and poured into petri dishes they were stored at temperature of 4ºC.

It is provided the culture media composition for volumes of 1 liter.

1.1. Luria-Bertani (LB) medium:

Peptone from casein	10.0 g	
Yeast extract	5.0 g	
Sodium chloride	10.9 g	(pH 7.0)

1.2. Mueller-Hinton (MH) Agar (acc. to CLSI)

Infusion from meat	2.0 g	
Casein-hydrolysate	17.5 g	
Starch	1.5 g	
Agar-agar	17.0 g	(pH 7.3)

2. General reagents and solutions

The composition of the buffer solutions of general use and of the loading dye used in electrophoresis is described below.

2.1. 1x Tris-Acetato EDTA (TAE) buffer (5 Prime, Deutschland)

2 M Tris-Acetate
0.05 M EDTA (pH 8.3)

(Note: "Stock" solution prepared at 50x.)

2.2. Tris-EDTA (TE)

10 M Tris-HCl (Sigma, USA)
1 mM EDTA (pH 7.5)

2.3. 6x Loading Dye (MBI Fermentas, Lithuania)

10 mM Tris-HCl
0.03% bromophenol blue
0.03% xylene cyanol FF
60% glycerol
60 mM EDTA (pH7.5)

3. Establishment of an E. coli collection

The detection of fecal pollution, sampling process and E. coli isolation and purification were performed in a previous study. (Araújo 2012; Araújo S. 2013). Following it is presented a brief summary of the procedures which conducted to the establishment of the E. coli collection used in the present study.

Analysis of the microbiological quality of water showed four time points which overcome the 500 E. coli /100 ml, the maximum allowable value that must be respected and not exceeded (Figure II.1.). Three of those time points corresponded to high-tide and one to low-tide. These events occurred in the beginning of the summer season and in the end of August in a week when the human affluence was low given the weather conditions verified. The better results of water quality occurred when the human affluence to the island is high. It was hypothesized that the microbiological quality of the beach water was related with the presence of seagulls that exist in less number when humans are present (Araújo 2012).

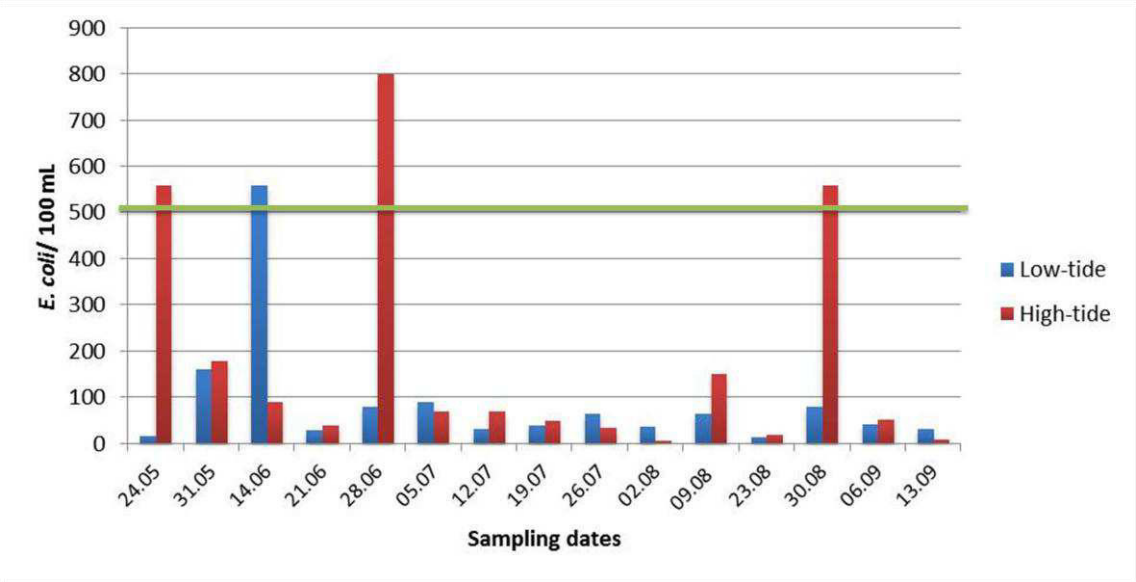


Figure II.1. – E. coli counting per 100 mL of water collected at low-tide and high tide moments (Araújo 2012).

To study this hypothesis and confirm the fecal pollution source, sampling was performed between May and September of 2011 and samples were: 1) water of the Berlenga beach, 2) seagull feces scattered in the beach and of the surrounding rocks, and 3) human-derived sewage from the island sanitary infrastructures.

Feces and sewage samples were subjected to homogenization in a saline solution and decimal dilutions were prepared. The resulting dilutions and water samples were subjected to filtrations in vacuum systems through 0.45 µm membranes to retain bacteria and filters were placed in Chromocult® Coliform Agar (CCA) (Merck, Germany) for incubation at 37ºC during 18 to 24 h.

For each source, E. coli strains were isolated by selecting the colonies with dark-blue to violet aspect. Each presumptive E. coli colony was streaked to CCA plates and incubated overnight at 37ºC as many times as necessary until pure cultures were obtained being then streaked from the selective media to Tryptic Soy Broth agar plates for maintenance of the collection. After E. coli confirmation with Kovac's indole reagent test, incubation in the differential MacConkey (Merck, Germany) agar medium and in mFC ((Merck, Germany) agar and 16S sequencing, pure cultures were cryopreserved at -80ºC.

The 939 recovered E. coli isolates were typed by BOX-PCR and the fingerprints were normalized and analyzed using the software GelCompar II ® software (Applied Maths, Belgium) to assess the genetic diversity of the E. coli library.

The analysis of the resulting dendrogram with an 85% similarity cutoff allowed the selection of the representative strains: banding patterns with more than 85% similarity were considered to represent isolates of the same strain and only one for each source was selected and included in the present study.

The selected isolates used for the present study were criopreserved in glycerol at -80ºC. The total collection analyzed during the present study comprised 414 isolates: 166 isolates from water samples, 179 isolates from feces, and 69 isolates from human-derived sewage samples.

4. Antibiotic susceptibility testing

The E. coli isolates were tested for susceptibility to 16 antibiotics by the disk diffusion method according to the procedure described next and established by the Clinical and Laboratory Standards Institute (CLSI) ([CLSI] 2012), using E. coli ATCC 25922 as a quality control strain. Antibiotic disks were obtained from Oxoid (Oxoid Ltd, Basingstoke, UK) and are listed below (Table II.1.).

Table II.1.- Antibiotics used for the susceptibility testing and the corresponding abbreviation according to the supplier (Oxoid Ltd, Basingstoke, UK) as well as the antibiotic concentration in the disk.

Classes	Antibiotics	Oxoid abr.	Disk conc. (µg)
Penicillins	Ampicillin	AMP	10
	Amoxicillin	AML	10
	Amoxicillin + Clavulanic Ac.	AMC	30
	Piperacillin	PRL	100
	Piperacillin + Tazobactam	TZP	110
1st gen. cephalosporins	Cefalotin	KF	30
2nd gen. cephalosporins	Ceftazidime	CAZ	30
	Cefotaxime	CTX	30
Aminoglycosides	Gentamicin	CN	10
	Streptomycin	S	10
Carbapenems	Imipenem	IMP	10
Quinolones	Nalidixic Ac.	NA	30
	Ciprofloxacin	CIP	5
Tetracycline		TE	30
Chloramphenicol		C	30
Sulfonamide + Trimethoprim		SXT	1,25/23,75

Agar antibiotic disk diffusion technique:

1- Prepare a bacterial suspension in sterile saline solution with a turbidity equivalent to half of grade one in McFarland scale (3-4 colonies);
2- Use the previous suspension to inoculate plates with MH Agar with a swab;
3- After drying apply the disks with antibiotic;
4- Incubate at 37ºC, 24 hours;
5- Measure the diameters of the circles of growth inhibition to each antibiotic;
6- Compare the measured diameters with the criteria established by the CLSI and classify each strain as sensitive, intermediate and resistant.

5. General conditions for Polymerase Chain Reaction amplification

PCR was performed with cell suspensions prepared in dH_2O using isolates streaked to LA medium and incubated at 37 ºC overnight. In each PCR experiment was used a negative control that differ from the reaction mixture by substituting the cell suspension by the same volume of dH_2O. The PCR reactions were performed in 25 µL volumes in a MyCycler Thermal Cycler (Bio-Rad, USA) and all primers used were ordered from Stab Vida, Portugal or from Sigma, USA. The programs for amplification are described in table II.2.. Reaction mixtures that were not immediately analyzed by electrophoresis were stored at -20ºC.

Table II.2. - PCR programs used for amplifications.

	PCR programs timings (x n)				
Temperature (ºC)	**A (x30)**	**B (x30)**	**C (x30)**	**D (x30)**	**E (x35)**
94	2'	5'	5'	10'	5'
94	15"	30"	30"	40"	30"
(x n) X	30"	30"	30"	40"	30"
72	45"	1'	30"	1'	1'
72	10'	7'	7'	7'	7'

X - the annealing temperature to each primer set; n – number of times the cycle is repeated; A, B, C, D and E – the five different PCR programs used in this study.

5.1. E. coli phylogenetic group determination

The phylogenetic group of each strain was determined according to Clermont et al, 2000 (Clermont, Bonacorsi et al. 2000) by multiplex PCR of the genes chuA and yjaA and the DNA fragment TspE4.C2 in the conditions showed in table II.3 using PCR program B (description in table II.2.) with an annealing temperature of 55ºC. PCR reagents were from NZYtech, Portugal. The strains were assigned to the phylogenetic groups as explained in table II.5.

Table II.3. - PCR primers for phylogroup determination.

Target	Amplicon Size (bp)	Primers Sequence (5' - 3')
chuA	279	chuA_F: GACGAACCAACGGTCAGGAT chuA_R: TGCCGCCAGTACCAAAGACA
yjaA	211	yjaA_F: TGAAGTGTCAGGAGACGCTG yjaA_R: ATGGAGAATGCGTTCCTCAAC
TspE4.C2	152	TspE4.C2_F: GAGTAATGTCGGGGCATTCA TspE4.C2_R: CGCGCCAACAAAGTATTACG

Table II.4. –One sample reaction mixture for PCR with NZYtech 2x Green Master Mix (NZYtech, Portugal).

NZYtech 2x Green Master Mix (2.5mM $MgCl_2$; 200 µM dNTPs; 0.2 U/µl DNA polymerase)	6.25 µl	
1µM Primer Forward	0.75 µl	(of each forward primer)
1µM Primer Reverse	0.75 µl	(of each reverse primer)
Cell suspension	1µl	
dH_2O	16.75 µl	

Table II.5. - Phylogroups genotypes (Clermont et al 2000).

	Fragments		
Phylogruops	**chuA**	**yjaA**	**TspE4.C2**
A_0	-	-	-
A_1	-	+	-
B1	-	-	+
$B2_2$	+	+	-
$B2_3$	+	+	+
D_1	+	-	-
D_2	+	-	+

Legend: (-) - negative result; (+) - positive result.

5.2. Screening for antibiotic resistance genes (ARGs)

Resistance genes were screened by PCR for isolates with resistance or intermediate phenotype to the antibiotics tested.

The reaction mixture used to amplify the ARGs is described in table II.4 Primers and reaction conditions to each target are described in table II.6.

Table II.6. - PCR primers and conditions for ARG amplifications (programs description is in TableII.2.).

Target	Amplicon Size (bp)	Program	Annealing temperature (ºC)	Primer Sequence (5'- 3')	Primers reference	Control Strains
bla_{OXA-48}	744	E	55	OXA_F: TTGGTGGCATCGATTATCGG OXA_R: GAGCACTTCTTTTGTGATGGC	(Poirel, Heritier et al. 2004)	Shewanella xiamenensis IR34 (Strain from the laboratory)
bla_{TEM}	425	B	44	TEM_F: AAAGATGCTGAAGATCA TEM_R: TTTGGTATGGCTTCATTC	(Speldooren, Heym et al. 1998)	K. pneumoniae 6T (Henriques, Fonseca et al. 2006)
bla_{SHV}	304	B	62	SHV_F: GCGAAAGCCAGCTGTCGGGC SHV_R: GATTGGCGGCGCTGTTATCGC	(Henriques, Moura et al. 2006)	K. pneumoniae 2s (Henriques, Fonseca et al. 2006)
bla_{CTX-M}	652	A	55	CTX_F: SCVATGTGCAGYACCAGTAA CTX_R: GCTGCCGGTYTTATCVCC	(Lu, Zhang et al. 2010)	K. pneumoniae Kp40 (Henriques, Fonseca et al. 2006)
bla_{IMP}	232	B	55	IMP_F: GAATAGAGTGGCTTAATTGTC IMP_R: GGTTTAAYAAAACAACCACC	(Henriques, Fonseca et al. 2006)	K. pneumoniae KP99c196 (Henriques, Fonseca et al. 2006))
bla_{VIM}	475	B	58	VIM_F: GATGGTGTTTGGTCGCATATCG VIM_R: GCCACGTTCCCCGCAGACG		P. aeruginosa NTU-39/00 (Henriques, Fonseca et al. 2006)
bla_{KPC}	538	B	55	KPC_F: CATTCAAGGGCTTTCTTGCTGC KPC_R: ACGACGGCATAGTCATTT	(Dallenne, Da Costa et al. 2010)	Klebsiella oxytoca Ko25 (Strain from the laboratory)
bla_{GES}	399	D	57	GES_F: AGTCGGCTAGACCGGAAAG GES_R: TTTGTCCGTGCTCAGGAT		K. pneumoniae 22K9 (Strain from the laboratory)
tet(A)	211	C	53	tetA_F: GCTACATCCTGCCTTC tetA_R: GCATAGATCGGAAGAG	(Nawaz, Sung et al. 2006)	E. coli M.I.10.2 (Henriques, Fonseca et al. 2008)
tet(B)	391	C	53	tetB_F: TCATTGCCGACCTCAG tetB_R: CCAACCATCACCATCC	(Nawaz, Sung et al. 2006)	E. coli M.I.10.1 (Henriques, Fonseca et al. 2008)
qnrA	521	C	53	qnrA_F: TTCTCACGCCAGGATTTG qnrA_R: CCATCCAGATCGGCAAA	(Guillard, Moret et al. 2011)	K. pneumoniae Kp 51 (Strain from the laboratory)
qnrB	261	C	53	qnrB_F: GGMATHGAAATTCGCCACTG qnrB_R: TTYGCBGYYCGCCAGTCG	(Cattoir, Poirel et al. 2007; Guillard, Moret et al. 2011)	K. pneumonia Kp1 (Santos, Caetano et al. 2011)
qnrS	428	C	54	qnrS_F: GCAAGTTCATTGAACAGGGT qnrS_R: TCTAAACCGTCGAGTTCGGCG	(Cattoir, Poirel et al. 2007)	K. oxytoca Ko25 (Strain from the laboratory)
sul1	239	C	50	sul1_F: CTGAACGATATCCAAGGATTYCC sul1_R: AAAAATCCCACGGRTC	(Heuer and Smalla 2007)	Aeromonas media (Moura, Oliveira et al. 2012)
sul2	293	C	60	sul2_F: GCGCTCAAGGCAGATGGCAT sul2_R: GCGTTTGATACCGGCACCCG	(Henriques, Fonseca et al. 2006)	E. coli A237 (strain from the laboratory)

5.2.1. Detection of $bla_{AmpC\text{-}like}$ genes by multiplex PCR

Isolates with resistance phenotype to 3rd generation cephalosporins were tested for plasmid mediated AmpC-like β-lactamases through a multiplex PCR technique targeting for $bla_{AmpC\text{-}like}$ genes according to Dallenne et al, 2000 (Dallenne, Da Costa et al. 2010), using the PCR program D and the annealing temperature of 60 ºC. Primers and their concentrations are described in table II.7. The reaction mixture is the same as listed in Table II.4. except for the primers concentration.

Table II .7. - Primers for $bla_{AmpC\text{-}like}$ multiplex PCR

Target	Amplicon Size (bp)	Primer Sequence (5' - 3')	[Primers] (pmol/µL)
bla_{ACC}	346	ACC_F: CACCTCCAGCGACTTGTTAC ACC_R: GTTAGCCAGCATCACGATCC	0.2
bla_{FOX}	162	FOX_F: CTACAGTGCGGGTGGTTT FOX_R: CTATTTGCGGCCAGGTGA	0.5
bla_{MOX}	895	MOX_F: GCAACAACGACAATCCATCCT MOX_R: GGGATAGGCGTAACTCTCCCAA	0.2
bla_{DHA}	997	DHA_F: TGATGGCACAGCAGGATATTC DHA_R: GCTTTGACTCTTTCGGTATTCG	0.5
bla_{CIT}	538	CIT_F: CGAAGAGGCAATGACCAGAC CIT_R: ACGGACAGGGTTAGGATAGY	0.2
bla_{EBC}	683	EBC_F: CGGTAAAGCCGATGTTGCG EBC_R: AGCCTAACCCCTGATACA	0.2

5.3. Determination of ARGs genomic context

The genomic context of $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CMY\text{-}2}$ was investigated by PCR and by sequencing the regions surrounding these genes. The two isolates with the $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CMY\text{-}2}$ genes were tested for the presence of the ISEcp1 insertion sequence and the region downstream the $bla_{CTX\text{-}M\text{-}1}$ was also characterized by testing for the presence of the Orf477 and the mucA gene. Primers were as described in table II.7 according with Eckert et al, 2006 and Saladin et al, 2002 (Saladin, Cao et al. 2002; Eckert, Gautier et al. 2006). Positive controls were performed with E. coli E24 (Tacao, Correia et al. 2012). The enzymes and reagents used in PCR are listed in tables II.2. and II.5.

Whenever necessary a thermal gradient PCR was performed testing different annealing temperatures to optimize the amplification.

Table II.8. - PCR conditions for genomic environment determination with the optimal annealing temperatures (programs description is in Table II.2.)

Target	Program	X (ºC)	Primer Sequence (5' - 3')
ISEcp1	D	50	ISEcp1_U1_F: AAAAATGATTGAAAGGTGGT CTX_R: GCTGCCGGTYTTATCVCC
	D	50	ISEcp1_5'_F: TTC AAAAAGCATAATCAAAGCC ISEcp1_U1_inv: ACCACCTTTCAATCATTTTT
	B	50	ISEcp1_U1_F: AAAAATGATTGAAAGGTGGT CIT_R: ACGGACAGGGTTAGGATAGY
	B	51	ISEcp1_U2_F: AATACTACCTTGCTTTCTGA CIT_R: ACGGACAGGGTTAGGATAGY
orf477	D	50	CTX_F: SCVATGTGCAGYACCAGTAA Orf477_R: ACTTCAAAAATTATGCCACC
mucA	D	50	Orf477_inv: GGTGGCATAATTTTTGAAGT mucA_R: GGCATCAGGCAGGGGTAAGG

X - the annealing temperature to each primer set

6. DNA electrophoresis and visualization

Amplicons were separated in a conventional electrophoresis at 80 V for 3h for phylogenetic group determination and 90 min for detecting ARGs and genomic context on a 1,5% (w/v) SeaKem® LE Agarose (Lonza, USA) gel in 1xTAE (5 Prime, Deutschland). In each gel it was used the DNA molecular weight marker GeneRuler^{TM} DNA Ladder Mix (MBI Fermentas, Lithuania) (Appendix A).

For visualization, the gels were stained for 10 min with a solution of 0.5 µg mL^{-1} ethidium bromide (Sigma, USA) and washed in dH_2O for 10 min. Gel images were captured under UV light with the imaging Molecular Imager® Gel Doc™ XR+ System (Bio-Rad, USA).

7. PCR products purification and sequencing

The amplicons were purified with the JetQuick® PCR Product Purification Spin Kit (Genomed, Germany) following the manufacturer instructions with some alterations as described next.

PCR products purification protocol:

1- Load: add 4 volumes of Binding Buffer to 1 volume of a PCR sample. After mixing pipet the sample into a JetQuick® Spin Column placed in a 2 mL Receiver Tube. Centrifuge the column at >12,000 x g for 1 min.

2- Wash: Re-insert the column into the empty Receiver Tube and add 500 µL of Wash Buffer (H2) with ethanol. Centrifuge the column at >12,000 x g for 1 min. Discard the flow-through placing the column in the same but empty Receiver Tube to centrifuge again at >12,000 x g for 1 min.

3- Elute: Place the column in to a 1.5 mL microcentrifuge tube. Add 35 µL of sterile water at 60 ºC to the column and centrifuge at >12,000 x g for 2 min.

4- Store: Store the purified DNA at -4ºC for immediate use or at -20ºC for long-term storage.

The PCR purified products were used as template in the sequencing reaction carried out by the company GATC (Germany).

The resulting sequences were edited with FinchTV program (Geopiza, USA). Online similarity searches were performed using the BLAST software at the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/).

8. Statistical Analysis

Principal component analysis (PCA, using a covariance matrix model) was carried out to explore the AR patterns of E. coli isolates, by reducing the multidimensional data matrix to an interpretable bidimensional biplot that explains the highest proportion of variation in the data. A similar approach was used in other studies (Parveen, Murphree et al. 1997; Su, Ying et al. 2012; Pereira, Santos et al. 2013). For this, AR phenotype and genotype data were a priori converted to a numeric code: each isolate x antibiotic combination was scored as 0 signifying sensitivity, 1 representing intermediate resistance and 2 representing resistance (phenotype data matrix); for the resistance genes tested 1 represented a positive PCR result for the gene and 0 a negative PCR result (genotype data matrix). The resulting data matrices met the assumptions of PCA, which were tested in SPSS.

One-way analyses of variance (ANOVA) were performed on the PCA sample scores to assess significant differences ($p \leq 0.05$) among sampling sites, phylogenetic groups and number of classes of resistance. This allowed testing the influence of these factors in terms of the isolates' AR profiles, without a priori defining an underlying data structure. ANOVAs and PCA were conducted using Minitab and CANOCO for Windows version 4.5 (Scientia Ltd., UK), respectively.

Redundancy analysis (RDA) was used to assess the amount of variation of the AR profiles of the isolates (phenotype data matrix) that was explained by the presence of resistance genes (genotype data matrix), isolate provenience (site), and taxonomic/phylogenetic group. Unlike PCA, RDA is a direct gradient analysis in the sense that assumes an underlying data structure, constrained by the selected explanatory matrices. Monte Carlo permutation tests (unrestricted permutations) were used to assess the significance of the models ($p \leq 0.05$). Genes with no positive PCR results were excluded from the RDA analyses.

III. RESULTS

1. E. coli isolates diversity

The 414 E. coli isolates selected for the present study were assigned to the main phylogenetic groups: A, B1, B2 and D. Considering the total collection from Berlenga, group A was the most prevalent (45.4%) followed by group B1 (30.9%) and group D (20.5%). The less prevalent group was B2 (3.4%).

In figure III.1. the differences in E. coli diversity between the three different sources (e.g. water, gull feces and human-derived sewage effluent) are shown. Groups A, B1 and D were present in all sources, while group B2 was only present in sewage (10.1%) and in a lower percentage in feces (3.9%). In all sources, group A was the more prevalent followed by group B1 and D.

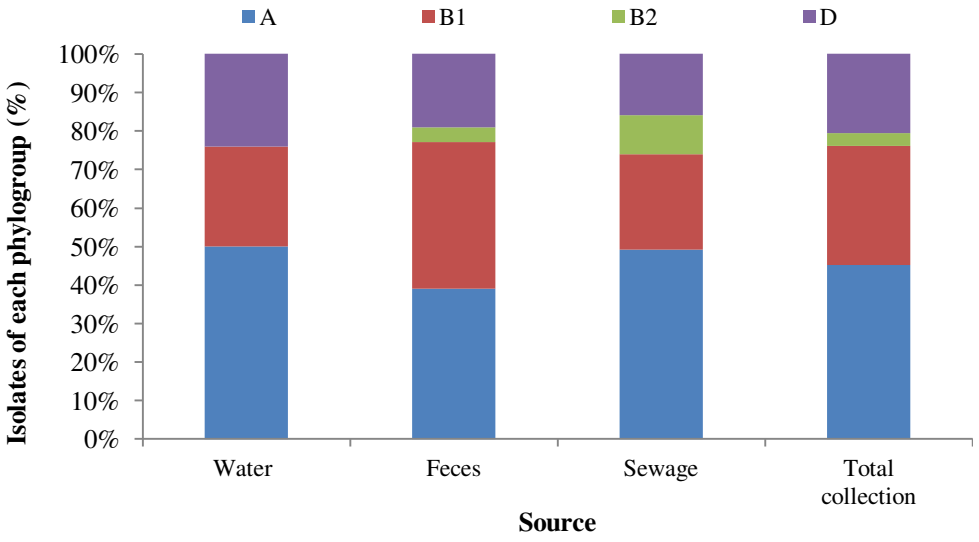


Figure III. 1.- Distribution of the main E. coli phylogenetic groups (A, B1, B2 and D) through the three sources and their prevalence in the total collection from Berlenga.

2. Antibiotics susceptibility test

Antimicrobial resistance phenotypes were determined for 414 E. coli isolates and the resistance frequencies to each antibiotic are presented in figure III.2. Isolates were classified as resistant, intermediate or susceptible as described on the section Materials and Methods but in the subsequent analysis of the results, isolates classified as intermediate were addressed as resistant. A total of 390 (94.2%) of the 414 isolates showed resistance to at least one of the antibiotics tested.

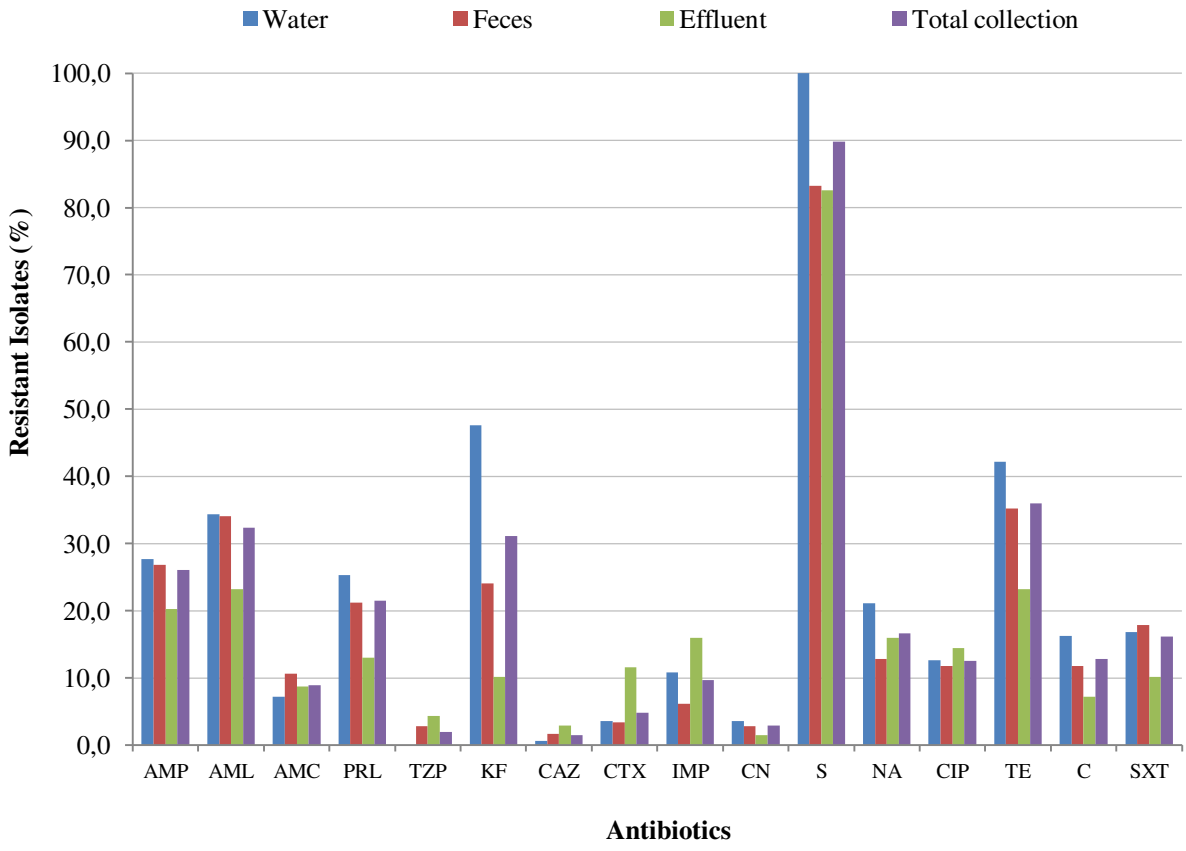


Figure III. 2. - Prevalence of resistant isolates to the antibiotics tested in the different sources and in the total collection. Antibiotics tested: AMP - ampicillin, AML- amoxicillin, AMC- amoxicillin + clavulanic acid, PRL- piperacillin, TZP-piperacillin + tazobactam, KF – Cefalotina, CAZ – Ceftazidime, CXT – Cefotaxime, CN – Gentamycin, S – Straptomycin, IMP- imipenem, NA- nalidixic acid, CIP-ciprofloxacin, TE-tetracyclin, C- Chloramphenicol, SXT- sulfonamide+trimethoprim.

Considering the total collection from the Berlenga Island, streptomycin and tetracycline were the antibiotics with the higher levels of resistance (89.9 and 36.0% of the total number of isolates, respectively), followed by amoxicillin (32.4%), cefalotin (31.2%), ampicillin (26.0%), piperacillin (21.5%) nalidixic acid (16.7%) and the combination sulfamethoxazole/trimethoprim (16.2%). Levels of resistance to ciprofloxacin and chloramphenicol were similar (12.6 and 12.8%, respectively). Berlenga isolates showed lower levels of resistance to imipenem (9.7%), the combination amoxicillin/clavulanic acid (8.9%), cefotaxime (4.8%), gentamicin (2.9%) and the combination piperacillin/tazobactam (1.9%). The antibiotic to which isolates were more susceptible was ceftazidime (0.7%).

Multiresistance (resistance to three or more classes of antibiotics) was present in 34.1% of the Berlenga isolates (figure III.3.). Multiresistance rates were higher among the water isolates and lower in the sewage effluent.

Figure III. 3. - Percentage of multiresistant isolates (resistant to three or more classes of antibiotics) in each source (W – water, F – feces, S –sewage) and considering the total collection (TC) from Berlenga.

All water isolates (n=166) were resistant to one or more antibiotics. Almost half (42.8%) of these isolates were resistant to two classes of antibiotics (figure III.4.) and 39% were multiresistant (Figure III.3.). All isolates were resistant to streptomycin. Nearly half of the isolates were resistant to cefalotin (47.6%) and tetracycline (42.2%). Resistance to penicillins was also frequent (34.3% for amoxicillin, 27.7% for ampicillin and 25.3% for piperacillin). Lower levels of resistance were detected to nalidixic acid (21.2%), the

combination sulfamethoxazole/ trimethoprim (16.9%) and chloramphenicol (16.3%). The 166 water isolates were susceptible to the combination piperacillin/tazobactam. Only 0.6% of water isolates were resistant to ceftazidime (corresponding to 1 isolate). Low levels of resistance were also detected to cefotaxime and gentamicin (3.6%), followed by ciprofloxacin (12.7%), imipenem (10.8%) and the combination amoxicillin/ clavulanic acid (7.2%) (Figure III.2.).

Susceptibility to the 16 antibiotics tested was verified in 10.5% of the feces isolates, the remaining being resistant to at least one antibiotic. Resistance to 2 classes of antibiotics is the phenotype with higher percentage of feces isolates (36.4%) as can be seen in figure III.4. In gull feces 32% of the isolates were multiresistant (Figure III.3.). From these isolates 83.2% were resistant to streptomycin. Apart from the aminoglycoside referred, resistances to tetracycline (35.2%) and amoxicillin (34,0%) were predominant, followed by ampicillin (26.8%), cefalotin (24.0%), piperacillin (21.2 %), sulfamethoxazole/ trimethoprim (17.9 %), the quinolones: nalidixic acid (12.8%) and ciprofloxacin (11.7%) and the combination amoxicillin/clavulanic acid (10,6%). Susceptibility to ceftazidime (1.7% of resistant isolates) was prevalent among feces isolates, as was susceptibility to piperacillin/tazobactam and gentamicin (2.8% of resistant isolates), cefotaxime (3.4% of resistant isolates) and imipenem (6.1% of resistant isolate) (Figure III.2.).

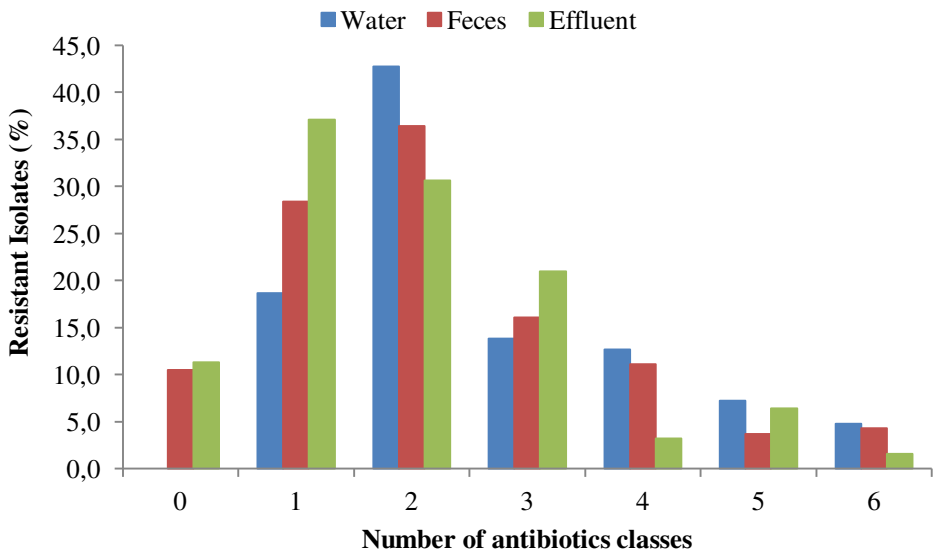


Figure III. 4. - Percentage of isolates from the three different sources resistant to a number of classes of antibiotics (0 classes means sensible to all antibiotics tested and resistance to 6 classes means resistance to all classes of antibiotics tested).

From the sewage isolates, 11.3% were sensible to the 16 antibiotics tested and the remaining were resistant to at least one antibiotic. Resistance to 1 class of antibiotics was the phenotype with higher percentage of isolates (37.1%) (Figure III.4.). From the human sewage 32% of the isolates were multiresistant. (Figure III.3.). Resistance to streptomycin was predominant (82.6%), followed by tetracycline and amoxicillin (23.2%), ampicillin (20.3%), nalidixic acid and imipenem (15.9%), ciprofloxacin (14.5%) and piperacillin (13.0%). It was prevalent among sewage isolates susceptibility to gentamicin (1.4% of resistant isolates), ceftazidime (2.9% of resistant isolates), tazobactam (4.3% of resistant isolates) and to chloramphenicol (7.2% of resistant isolates). The combinations amoxicillin/ clavulanic acid (8.7%) and sulfamethoxazole/ trimethoprim (10.1%) showed, comparatively with the levels of resistance to the other antibiotics, intermediate levels of resistance (Figure III.2.).

Analysing the levels of resistance in water, gull feces and human sewage, resistance to all groups of antibiotics tested were found as also higher levels of resistance to penicillins (except for the combinations of penicillins and β-lactamase inhibitors amoxicillin/clavulanic acid and piperacillin/tazobactam), aminoglycosides (streptomycin) and tetracyclines in the 3 sources. Resistance to 3rd generation cephalosporins and imipenem was rare and was mainly found in isolates from the sewage effluent (the prevalence in the sewage is nearly three times higher than in the feces and water) (Figure III.5.). Resistance to ciprofloxacin and the combination piperacillin/tazobactam was also higher in the sewage than in water and gull feces (Figure III.5.). To half of the antibiotics used, which are the penicillins ampicillin, amoxicillin, piperacillin, 1st generation cephalosporin cefalotin, the aminoglycoside gentamicin, tetracycline, chloramphenicol and the combination sulfamethoxazole/ trimethoprim resistance levels in the sewage are lower than in water and feces having these two sources more close levels of resistance for these antibiotics (Figure III.2.).

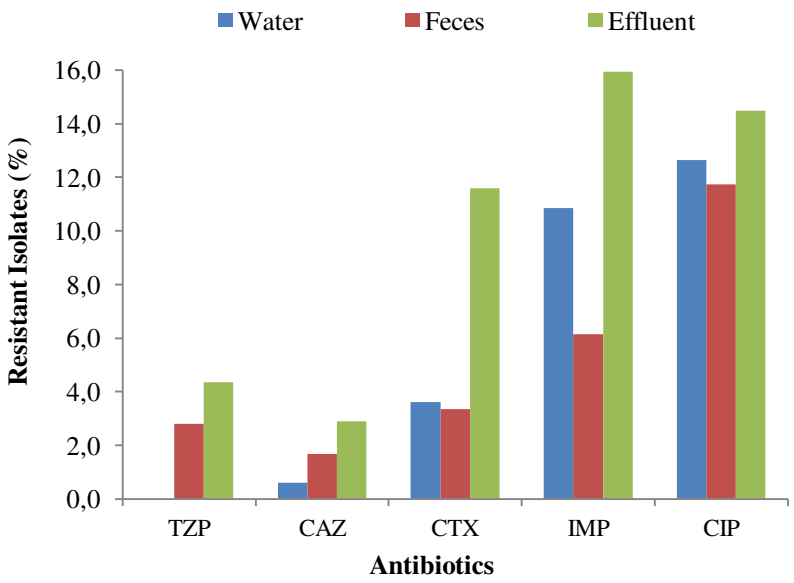


Figure III. 5.- Prevalence of resistant isolates (in detail from figure III.2) for five last line antibiotics used preferentially in humans: the combination piperacillin/tazobactam (TZP), the 3rd generation cephalosporins ceftazidime (CAZ) and cefotaxime (CTX), the carbapenem imipenem (IMP) and the fluoroquinolone ciprofloxacin (CIP).

There were a total of 126 different phenotypes for the 390 isolates with resistance to at least one of the antibiotics tested. From the 126 different phenotypes, 91 are represented by a single isolate. In table III.1. are listed the 35 phenotypes represented by two or more isolates and the respective phylogenetic groups. Of the 390 isolates with resistance to at least one antibiotic, 264 (67.8%) are represented in the listed phenotypes. From the 35 phenotypes, 15 (42.9%) are multiresistant phenotypes. The most prevalent is monoresistance to streptomycin (21,7%), followed by resistance to both streptomycin and tetracycline (7,2%), cefalotin and streptomycin (6,5%) and the phenotype of resistance to amoxicillin, cefalotin and streptomycin (2,7%).

Table III. 1 - Distribution of the resistance phenotypes and phylogenetic groups through the three sources - in grey the most prevalent. (antibiotics description in Index of Abreviations)

Phenotype	Water (n=166)					Feces (n=179)					Sewage effluent (n=69)					Total (%)
	A	B1	B2	D	Total	A	B1	B2	D	Total	A	B1	B2	D	Total	
S	17	2	0	12	31	18	12	2	7	39	9	5	1	5	20	90 (21,7)
AMC, S	1	0	0	0	1	0	1	0	1	2	0	0	0	0	0	3 (0,7)
AML, S	1	0	0	0	1	0	3	0	0	3	1	0	0	0	1	5 (1,2)
AMP, S	0	0	0	1	1	0	0	0	0	0	2	0	1	0	3	4 (1,0)
CTX, S	0	0	0	0	0	0	0	0	1	1	0	1	0	0	1	2 (0,5)
IPM, S	1	2	0	1	4	2	0	0	2	4	0	0	0	0	0	8 (1,9)
KF, S	11	6	0	5	22	1	0	0	4	5	0	0	0	0	0	27 (6,5)
S, CIP	0	0	0	0	0	1	1	0	0	2	1	0	0	0	1	3 (0,7)
S, NA	2	2	0	0	4	0	0	0	1	1	0	0	0	1	1	6 (1,4)
S, STX	0	0	0	0	0	1	1	0	0	2	1	0	0	0	1	3 (0,7)
S, TE	11	2	0	1	14	11	0	0	2	13	1	1	0	1	3	30 (7,2)
AML, IPM, S	0	0	0	0	0	0	1	0	0	1	0	1	0	0	1	2 (0,5)
AML, KF, S	0	5	0	1	6	0	4	0	0	4	0	1	0	0	1	11 (2,7)
AMP, AML, S	0	0	0	0	0	2	2	0	0	4	0	1	0	1	2	6 (1,4)
CN, S, TE	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	2 (0,5)
S, NA, CIP	3	0	0	0	3	0	0	0	0	0	0	0	0	0	0	3 (0,7)
S, NA, TE	0	2	0	0	2	1	0	0	0	1	0	0	0	1	1	4 (1,0)
S, TE, STX	1	1	0	0	2	0	1	0	0	1	0	0	0	0	0	3 (0,7)
KF, IPM, S	1	0	0	1	2	0	0	0	0	0	0	0	0	0	0	2 (0,5)
KF, S, NA	0	2	0	1	3	0	0	0	0	0	0	0	0	0	0	3 (0,7)
KF, S, TE	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	2 (0,5)
AMP, AML, PRL, S	0	1	0	0	1	0	1	0	1	2	0	0	0	0	0	3 (0,7)
AMP, AML, KF, S	0	0	0	1	1	0	1	0	1	2	0	0	0	0	0	3 (0,7)
AML, KF, S, TE	0	0	0	2	2	0	0	0	1	1	0	0	0	0	0	3 (0,7)
AMP, AML, PRL, S, TE	2	0	0	0	2	1	0	0	0	1	1	0	0	0	1	4 (1,0)
AMP, AML, AMC, KF, S	0	0	0	0	0	0	2	0	0	2	0	0	0	0	0	2 (0,5)
AMP, AML, PRL, S, TE, C	1	0	0	2	3	0	0	0	0	0	0	0	0	0	0	3 (0,7)
AMP, AML, PRL, KF, S, TE	1	1	0	0	2	1	2	0	0	3	1	0	0	0	1	6 (1,4)
AMP, AML, PRL, S, TE, STX	2	0	0	0	2	0	0	0	1	1	0	0	0	0	0	3 (0,7)
AMP, AML, PRL, S, TE, C, STX	0	0	0	0	0	2	0	0	1	3	0	0	0	0	0	3 (0,7)
AMP, AML, PRL, KF, S, TE, STX	1	1	0	0	2	1	1	0	2	4	0	0	0	0	0	6 (1,4)
AMP, AML, PRL, S, CIP, TE, C	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	2 (0,5)
AMP, AML, AMC, PRL, KF, S, TE, STX	1	0	0	1	2	0	0	0	0	0	0	0	0	0	0	2 (0,5)
AMP, AML, AMC, PRL, IPM, S, TE, C, STX	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	2 (0,5)
AMP, AML, AMC, PRL, KF, S, TE, C, STX	1	0	0	0	1	1	0	0	0	1	0	1	0	0	1	3 (0,7)
Total no of isolates with resistance phenotype	60	27	0	31	118	44	33	2	27	106	18	11	2	9	40	264 (63,8)

Considering the different sources and excluding monoresistance to streptomycin (Figure III.6.) the most prevalent phenotype in water isolates was cefalotin and streptomycin (KF, S) with a prevalence of 13.3% followed by streptomycin and tetracycline resistance (S, TE) with 8.4%, resistance to amoxicillin, cefalotin and streptomycin (AML, KF, S) with 3.6% and resistance to imipenem and streptomycin (IMP, S) with 2.4%. In feces isolates streptomycin and tetracycline resistance phenotype (S, TE) was the second most prevalent (7.3 %), followed by cefalotin and streptomycin resistance (KF, S) with 2.8% of prevalence and with a prevalence of 2.2% the phenotypes of resistance to imipenem and streptomycin (IMP, S), amoxicillin, cefalotin and streptomycin (AML, KF, S), ampicillin, amoxicillin and streptomycin (AMP, AML, S) and seven antibiotic phenotype AMP, AML, PRL, KF, S, TE and SXT. The sewage phenotypes with higher prevalence (4.3%) after streptomycin were ampicillin and streptomycin (AMP, S) and streptomycin and tetracycline (S, TE) followed by the three antibiotic phenotype AML, KF, S with a prevalence of 2.7%.

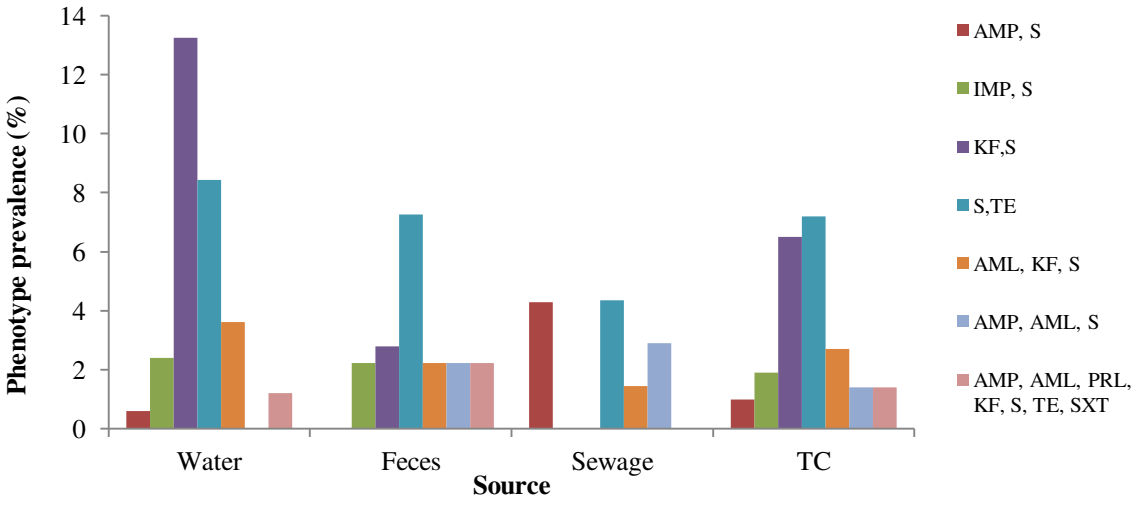
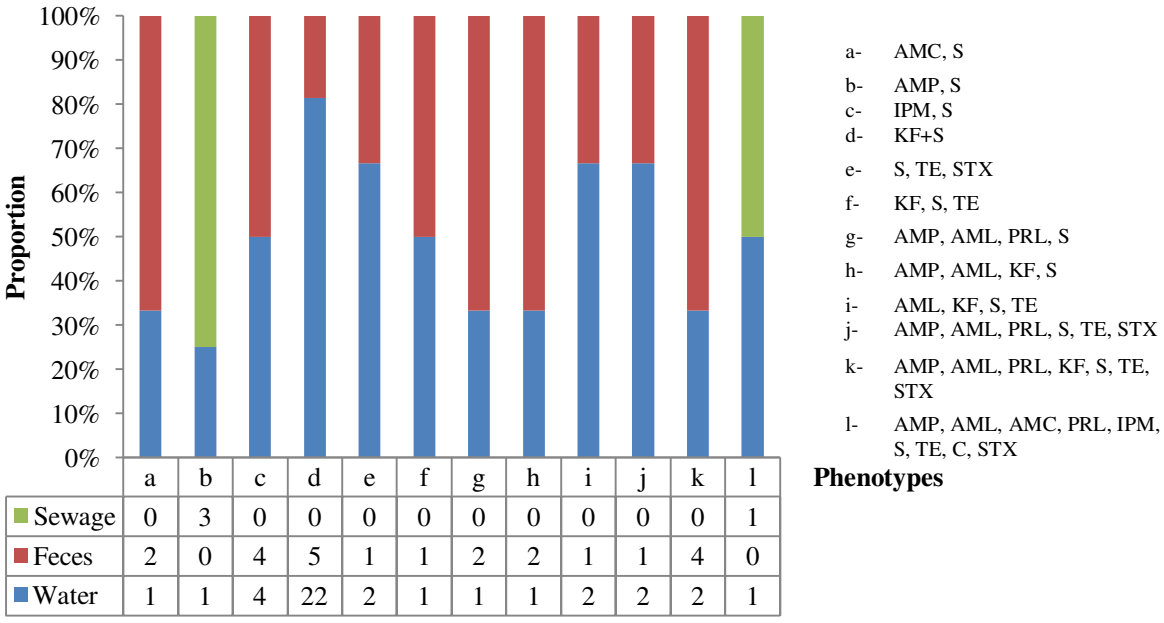


Figure III. 6.- Most prevalent phenotypes in the different sources and in the total collection (TC).

From table III.1. phenotypes only present simultaneously in isolates from water and feces or in isolates from water and sewage were selected (Figure III.7). There were ten different phenotypes common to water and gull feces while only two phenotypes were common to water and sewage isolates.



	a	b	c	d	e	f	g	h	i	j	k	l
Sewage	0	3	0	0	0	0	0	0	0	0	0	1
Feces	2	0	4	5	1	1	2	2	1	1	4	0
Water	1	1	4	22	2	1	1	1	2	2	2	1

Figure III. 7.- Comparision of the proportions of isolates in two sources and with respect to phenotypes that were present only in water and feces or in water and in the sewage effluent.

2.1. Antimicrobial resistance vs. phylogenetic group

The analysis of the antimicrobial resistance over phylogenetic groups showed that isolates included in group D were more frequently resistant to the tested antibiotics (96.5% of the D isolates were resistant to at least one antibiotic) followed by isolates included in group A (95.7%). On the other hand, 57.1% of B2 were susceptible to the antimicrobial compounds. However comparisons with this phylogenetic group should be cautious since only 14 isolates were included in this group. Distribution of phylogenetic groups according to the number of classes of antibiotics to which isolates were resistant is presented in figure III.8.: isolates of group B2 were resistant to less antibiotic classes and no isolate of this group was resistant to five or six classes of antibiotics.

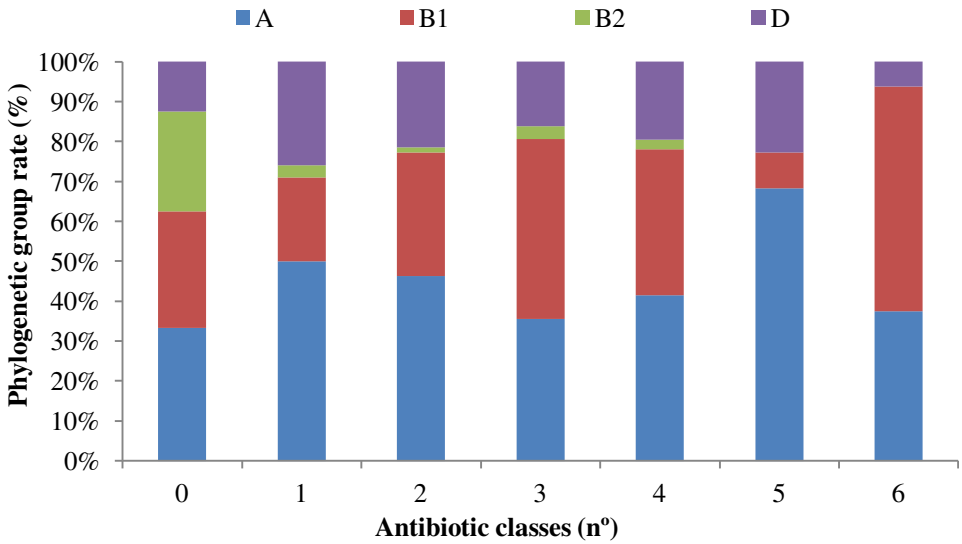


Figure III. 8. - Phylogenetic group rate distribution among classes of number of antibiotic resistant phenotypes.

Selecting the isolates with resistance to imipenem, an antibiotic used preferentially in humans, it was observed a prevalence of 21.9% of B1 isolates followed by the 7.1% of B2 isolates, 4.8% of A isolates and 4.7% of D isolates.

2.2. Statistical analysis of the AR phenotypes

The PCA biplot representing the distribution of isolates according to their AR profiles is presented in Figure III.9. The axes represent the extracted principal components, with only the first two being plotted, as they explain most of the variation in the data (first component, 44.1%; second component, 9.7%).

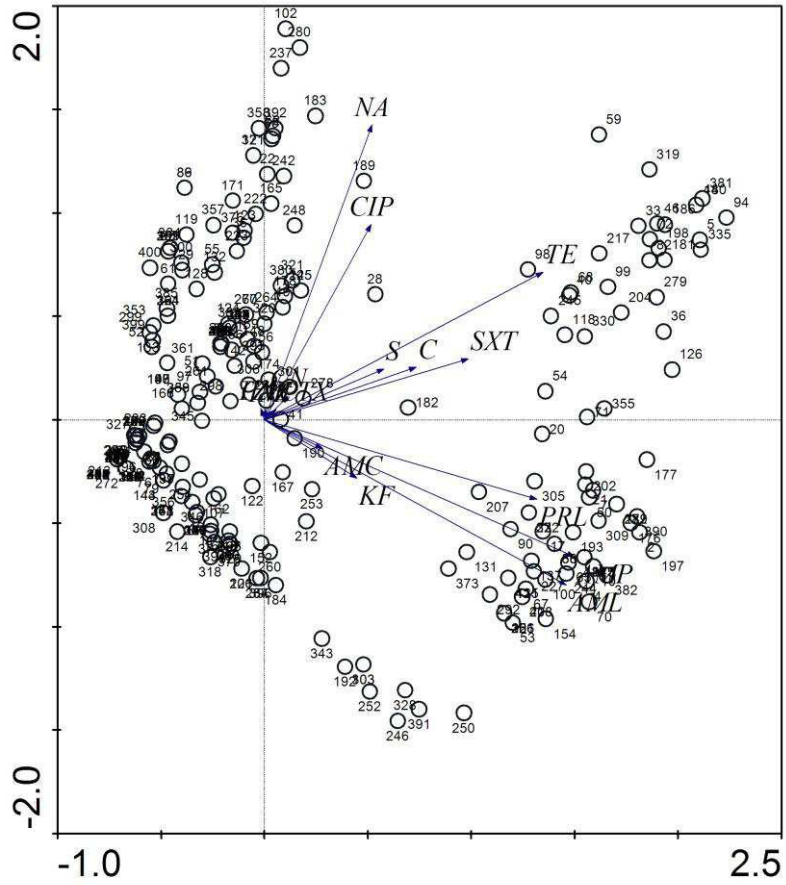


Figure III. 9 – PCA biplot of E. coli isolate scores (white circles, and corresponding isolate number) in terms of their AR profile (arrows represent phenotypes of resistance). Axes represent first (44.1%) and second principal component (9.7%).

It is possible to see at the bottom right quadrant a group of isolates scores that is justified by the common resistance to the penicillins AMP, AML and PRL. Some of the antibiotics with higher influence in the scores distribution correspond to those for which higher levels of resistance were registered, like the penicillins AMP, AML and PRL and tetracycline. The antibiotics to which resistance was rare have lower influence in the distribution in PCA analyzes, like imipenem, cefotaxime and gentamycin.

Significant differences in PC1 scores were found among sites [ANOVA: $F(2, 411) = 3.7$, $p = 0.025$], due to a significantly higher PC1 score in seawater isolates comparatively to sewage isolates (Tukey test, $p < 0.05$). On the contrary, no significant spatial differences were found for PC2 scores [ANOVA: $F(2, 411) = 1.2$, $p = 0.312$].

Significant differences in terms of AR were found across phylogenetic groups [ANOVA: $F(3, 410) = 2.8$, $p = 0.038$], with group B1 bearing a significantly higher PC1 score than B2 (Tukey test, $p < 0.05$). However, as previously highlighted, the number of isolates classified as B2 was much lower than the number of isolates included in other phylogenetic groups. Again, no significant differences were found for PC2 [ANOVA: $F(3, 410) = 0.41$, $p = 0.744$].

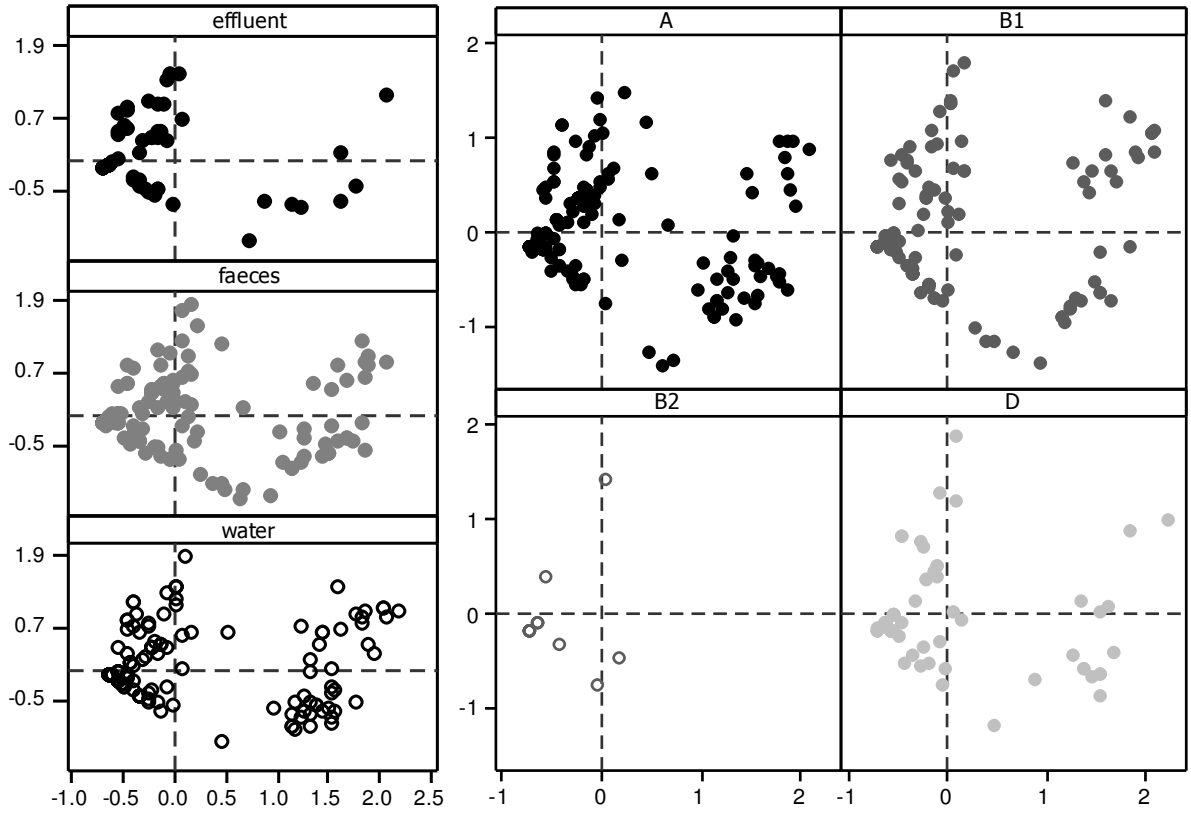


Figure III. 10. – PCA biplot (from figure III.9.) of E. coli isolate scores (circles) in terms of their AR profile (arrows were removed to facilitate visualization). Isolate scores are depicted according to site of origin (left panel) or phylogenetic group (A, B1, B2, D). Axes represent first (44.1%) and second principal component (9.7%).

The most striking pattern in the AR profile was revealed when analyzing the variation of PC1 and PC2 scores relatively to the number of classes that isolates were resistant to (Figure III.11 and 12). Significant differences were found in terms of PC1 [ANOVA: $F(6, 407) = 280$, $p < 0.001$] and PC2 [ANOVA: $F(6, 407) = 10.7$, $p < 0.001$] scores. This confirms an AR gradient, with less resistant isolates consistently displaying lower PC1 scores (i.e., being located at the left of the biplot) and more resistant isolates displaying higher PC1 scores (i.e. isolates resistant to six classes of antibiotics being placed at the upper right quadrant of the biplot). Differences are not so pronounced for PC2 scores.

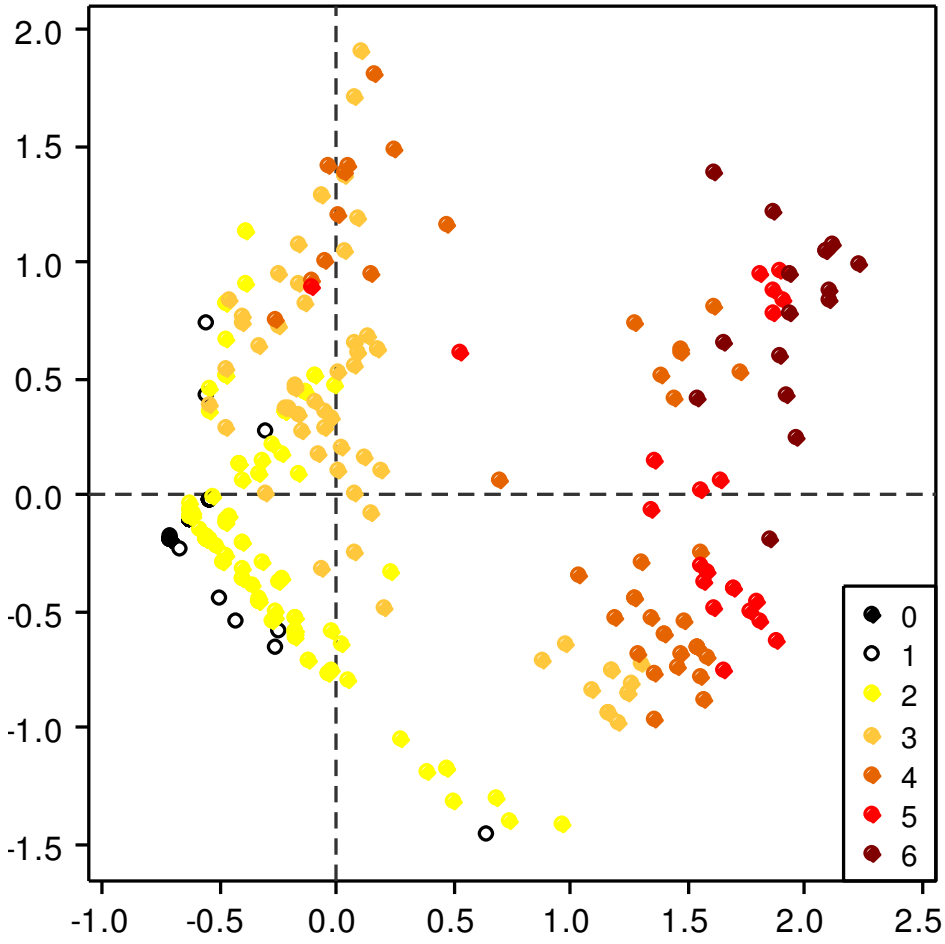


Figure III. 11. – PCA biplot (from figure III.9.) of E. coli isolate scores (circles) in terms of their AR profile (arrows were removed to facilitate visualization). Colors represent the number of antibiotic classes that isolates are resistant to (0-6). Axes represent first (44.1%) and second principal component (9.7%).

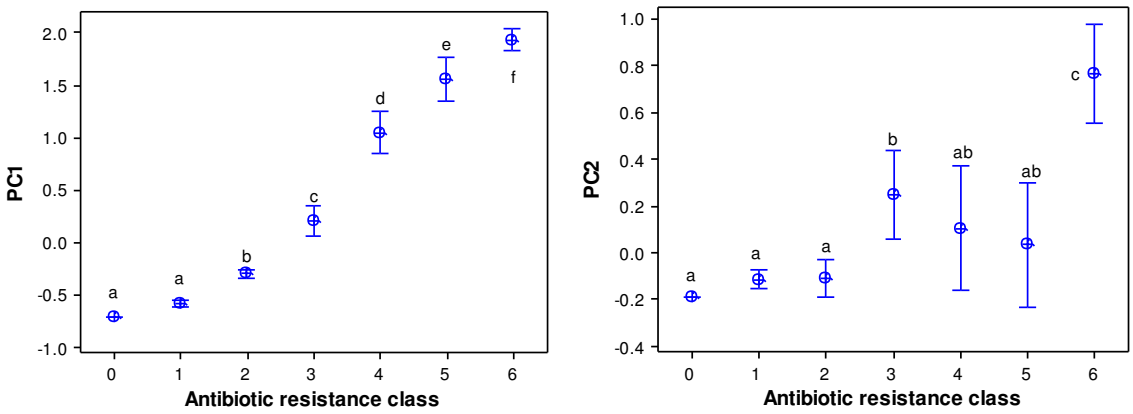


Figure III. 12. AR profiles of E. coli isolates: variation of first (PC1, left) and second principal (PC2, right) components extracted from PCA across resistance classes (number of antibiotic classes that isolates were resistant to). Lowercase letters represent statistically significant differences among resistance classes (Tukey test, $p < 0.05$).

3. Antibiotic resistance genes

Resistance genes were screened for isolates with resistance or intermediate phenotype to the antibiotics listed in table III.2.

Table III. 2 – Prevalence of resistance genes detected in E. coli isolates from the different sources over the number of strains that showed resistance to at least one compound of the antibiotic family in question.

Antibiotics	Tested genes	Water (n=166)		Feces (n=179)		Sewage (n=69)	
		No of isolates w/ resistance phenotype	Positive PCR results (%)	No of isolates w/ resistance phenotype	Positive PCR results (%)	No of isolates w/ resistance phenotype	Positive PCR results (%)
Penicillins (AMP, AML, AMC, PRL, TZP) & **3rd generation Cephalosporins** (CAZ, CTX)	bla_{TEM}	68	69.1	82	37.8	33	24.2
	bla_{SHV}		1.5		0		0
3rd generation Cephalosporins (CAZ, CTX)	$bla_{CTX-M-1}$	6	16.6	8	0	8	0
	bla_{CMY-2}		0		12.5		0
Carbapenems (IMP)	bla_{OXA-48}	18	0	11	0	11	0
	bla_{IMP}		0		0		0
	bla_{VIM}		0		0		0
	bla_{GES}		0		0		0
	bla_{KPC}		0		0		0
Quinolones (NA, CIP)	qnrA	39	0	30	0	17	0
	qnrB		2.5		6.7		0
	qnrS		5.1		16.7		5.8
Tetracycline (TE)	tet(A)	70	68.6	63	69.8	16	31.3
	tet(B)		31.4		23.8		37.5
Sulfamethoxazole/ trimethoprim (SXT)	sul1	28	28.6	32	25.0	7	28.6
	sul2		64.3		59.4		42.9

Isolates with resistance to the penicillins and 3rd generation cephalosporins were tested for bla_{TEM} and bla_{SHV} genes: water isolates were the ones with higher percentage of positive PCR results (69.1%), while feces and sewage isolates had less than half of positive

PCR results (37.8 and 24.2%, respectively); the gene bla_{SHV} was only present in one isolate from water. 3rd generation cephalosporins resistant isolates were also tested for bla_{CTX-M} and $bla_{AmpC-like}$ genes: one isolate from water had a $bla_{CTX-M-1}$ gene and one isolate from gull feces had a bla_{CMY-2} gene. Isolates with resistance to imipenem were tested for bla_{OXA-48}, bla_{IMP}, bla_{VIM}, bla_{GES} and bla_{KPC} but these genes were not detected with the primers and PCR conditions used.

Quinolone resistant isolates were tested for qnr genes A, B and S: no isolate had qnrA gene; qnrB genes were only present in water (2.5% of positive PCR results) and in feces (6.7%); qnrS was the quinolone resistance gene tested with higher percentage of positive PCR results for all sources with 5.1% and 5.8% for water and sewage isolates respectively and 16.7% for feces isolates. Feces isolates were the ones with higher percentage of qnrB and S genes (6.7 and 16.7 %).

tet(A) and tet(B) were the genes tested for tetracycline resistant isolates. The tet genes tested were found in isolates from the three sources. tet(A) genes were predominant in water (68.6% of positive PCR results) and feces isolates (69.8%) in respect to tet(B) genes (31.4% in water and 23.8% in feces). The sewage and water isolates had a similar percentage of tet(B) (37.5%) and tet(A) genes (31.3%). One isolate from water and another from gull feces presented the two genes, tet(A) and tet(B).

The three sources presented isolates with sul1 and sul2 genes. sul2 genes were in higher percentage in respect to sul1 in all isolates sources. Water and sewage isolates had the same percentage of sul1 genes (28.6%) and feces isolates had 25.0% of positive PCR results for this gene. For sul2 gene 64.3% of water isolates had positive PCR results, 59.4% of feces isolates and 42.9% of the sewage isolates. From the isolates resistant to sulfamethoxazole/trimethoprim both sul1 and sul2 genes were detected in 21.4% of water isolates, 18.6% of seagull feces isolates and 28.6% of the sewage isolates.

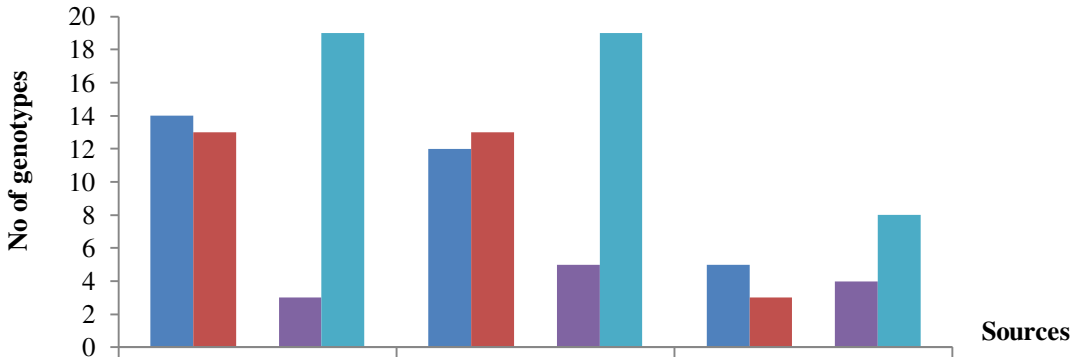
In table III.3., are listed the genotypes detected and the distribution of isolates among phylogenetic groups: 40.3% (n=157) of the 390 isolates with resistance phenotypes had at least one of the resistance genes tested; the most prevalent genotype was tet(A) (11,3%) followed by bla_{TEM} + tet(A) (5.4%), tet(B) (4.4%) and bla_{TEM} + tet(B) (3.1%).

Table III. 3 - Distribution of the resistance genes and phylogenetic groups through the three sources (highlighted in grey the most prevalent genotypes).

Genotype	Water (n=166)					Feces (n=162)					Sewage effluent (n=62)					Total (n=390) (%)
	A	B1	B2	D	Total	A	B1	B2	D	Total	A	B1	B2	D	Total	
bla_{CMY-2}	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1 (0.3)
bla_{TEM}	2	1	0	0	3	1	2	0	1	4	1	0	0	0	1	8 (2.1)
tet(A)	**13**	**3**	**0**	**5**	**21**	**15**	**2**	**0**	**3**	**20**	**2**	**0**	**0**	**1**	**3**	**44 (11.3)**
tet(B)	**4**	**3**	**0**	**0**	**7**	**5**	**0**	**0**	**3**	**8**	**1**	**0**	**0**	**1**	**2**	**17 (4.4)**
qnrB	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1 (0.3)
qnrS	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	2 (0.3)
sul1	0	1	0	0	1	0	3	0	0	3	0	0	0	0	0	4 (1.0)
sul1 + sul2	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1 (0.3)
tet(A) + qnrB	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1 (0.3)
tet(A) + sul1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1 (0.3)
tet(A) + sul2	0	1	0	0	1	2	0	0	0	2	0	0	0	0	0	3 (0.8)
bla_{TEM} + tet(A)	**3**	**4**	**0**	**6**	**13**	**3**	**3**	**0**	**0**	**6**	**0**	**1**	**0**	**1**	**2**	**21 (5.4)**
bla_{TEM} + tet(B)	**6**	**1**	**0**	**0**	**7**	**1**	**2**	**0**	**0**	**3**	**2**	**0**	**0**	**0**	**2**	**12 (3.1)**
bla_{TEM} + sul2	0	1	0	0	1	0	1	0	0	1	0	0	0	1	1	3 (0.8)
bla_{TEM} + tet(A) + tet(B)	0	1	0	0	1	1	0	0	0	1	0	0	0	0	0	2 (0.5)
bla_{TEM} + tet(A) + qnrS	1	0	0	0	1	2	0	0	0	2	0	0	0	0	0	3 (0.8)
bla_{TEM} + tet(A) + qnrB	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1 (0.3)
bla_{TEM} + tet(A) + su11	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1 (0.3)
bla_{TEM} + tet(A) + sul2	2	0	0	2	4	1	2	0	2	5	0	0	0	0	0	9 (2.3)
bla_{TEM} + tet(B) + qnrS	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1 (0.3)
bla_{TEM} + tet(B) + sul1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1 (0.3)
bla_{TEM} + tet(B) + sul2	2	2	0	0	4	0	1	0	0	1	0	0	0	0	0	5 (1.3)
bla_{TEM} + tet(A) + sul1 + sul2	3	1	0	0	4	0	3	0	2	5	0	0	0	0	0	9 (2.3)
bla_{TEM} + tet(B) + sul1 + sul2	1	1	0	0	2	0	0	0	0	0	1	1	0	0	2	4 (1.0)
bla_{TEM} + bla_{SHV} + tet(A) + sul2	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1 (0.3)
bla_{TEM} + $bla_{CTX-M-1}$ + tet(A) + qnrS	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1 (0.3)
bla_{TEM} + tet(B) + qnrS + sul1 + sul2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1 (0.3)
Total no of isolates with resistance genes	41	21	0	13	75	34	23	0	11	68	7	3	0	4	14	157 (40.3)

There were a total of 27 different genotypes (table III.3.). Figure III.13. shows their distribution among the different sources and, in each source, the percentage of different genotypes for the main phylogenetic groups. From the total of 27 different genotypes, 19 (70.1%) different genotypes were counted in the water, 19 (70.1%) in feces and 8 (29.6%) in the effluent isolates. Group A and B1 in water and feces sources are the ones with higher number of genotypes: for the water source 14 different genotypes were counted in group A isolates and 13 in group B1; in feces 12 genotypes were counted for group A isolates and 13 for group B1.In the effluent source A (n=5) and D (n=8) groups are the ones with higher number of different genotypes. Isolates from group B2 did not have positive PCR results for the screened ARGs showing no genotypes.

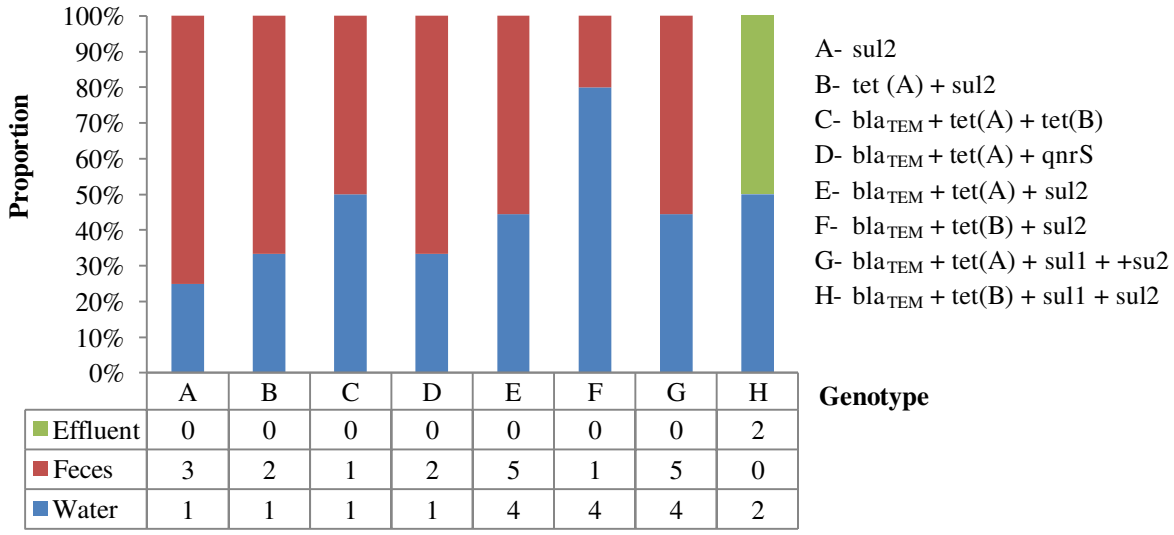
Considering only the main phylogenetic groups, A isolates had a total of 19 genotypes, group B1 18 genotypes and group D a total of 7 genotypes, being and A and B1 the groups with higher number of different genotypes.



	Water	Feces	Effluent
A	14	12	5
B1	13	13	3
B2	0	0	0
D	3	5	4
Total	19	19	8

Figure III. 13. – Number of the different genotypes for each phylogenetic group among each source and the total number of genotypes in each source.

From table III.3. genotypes common to water and feces or water and sewage were selected. The genotypes selected and their proportion and number in each source are in figure III.14. There are 7 different genotypes present only in water and in gull feces and there is one genotype in water that exists only in the sewage isolates: bla_{TEM} + tet (B) + sul1 + sul2.



	A	B	C	D	E	F	G	H
Effluent	0	0	0	0	0	0	0	2
Feces	3	2	1	2	5	1	5	0
Water	1	1	1	1	4	4	4	2

Figure III. 14. - Proportion between the two sources of the genotypes selected that existed only in water and feces isolates or in water and in the sewage isolates.

The genes bla_{TEM} and bla_{SHV} amplified from isolates with resistance to 3rd generation cephalosporins were sequenced as listed in table III.4 to determine the encoded enzyme variant. The 3 water isolates had the ampicillin resistance gene bla_{TEM-1} and isolate A7 had also the ESBL encoding gene $bla_{SHV-12.}$ Also the genes bla_{CTM-X} and $bla_{AmpC-like}$ were sequenced and identified (100% identity) as $bla_{CTX-M-1}$ and bla_{CMY-2} respectively.

Genes amplified with QnrB and QnrS primers were all identified as qnrB19 and qnrS1 (100% identity).

Table III. 4. – Sequenced genes and the corresponding isolates and phylogenetic group.

ARGs	Isolates (phylogroup)
bla_{TEM-1}	A7 (A), A9(A), A237 (A)
bla_{SHV-12}	A7 (A)
$bla_{CTX-M-1}$	A237(A)
bla_{CMY-2}	F155 (A)
qnrB19	A100 (A), F84 (A), F257 (B1)
qnrS1	A152 (A), A237 (A), F74 (B1), F317 (A), F368 (A), E80 (B1)

3.1. Genomic context of the $bla_{CTX-M-1}$ and bla_{CMY-2} genes

In Figure III.12 and 13 is presented a schematic representation of the genomic environments of the $bla_{CTX-M-1}$ (annotation in appendix B.1.) and bla_{CMY-2} (annotation in appendix B.2.) genes. The insertion sequence ISEcp1 was found in the upstream region of both genes. The distance between ISEcp1 and the start codon of bla genes was 80 bp for $bla_{CTX-M-1}$ and 323pb to bla_{CMY-2}. The $bla_{CTX-M-1}$ presented Orf477 downstream.

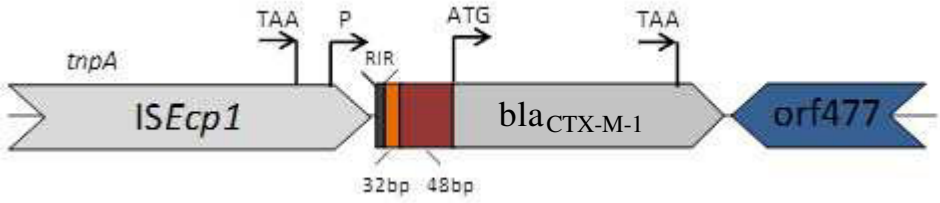


Figure III. 15. – E. coli strain A237 insertion sequence ISEcp1, partial sequence; β-lactamase CTX-M-1 ($bla_{CTX-M-1}$) gene, complete cds; and hypothetical protein gene, partial sequence; Length=1176 (tnpA – transposase A; RIR – right inverted repeat; P-promoter).

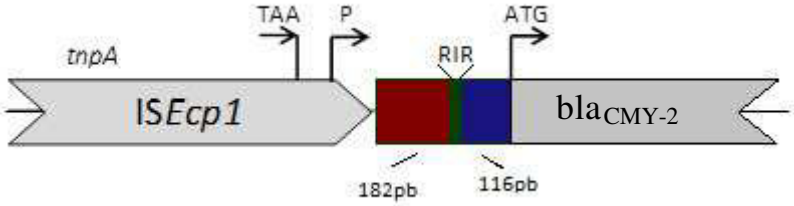


Figure III. 16.- E. coli strain F155 insertion sequence ISEcp1, partial sequence; β-lactamase CMY-2 (bla_{CMY-2}) gene, partial sequence; Length=1017 (tnpA – transposase A; RIR – right inverted repeat; P-promoter).

3.2. Statistical analysis for the AR genotypes.

PCA biplot is in Figure III.17. and the axes represent the extracted principal components, with only the first two being plotted, as they explain most of the variation in the data 70,8% The number of scores in the ARGs profile of the PCA analysis are in accordance with the number of genotypes observed (n=27). The genes with higher influence in the distribution of the isolates scores correspond to the genes detected most frequently bla_{TEM}, tet(A), tet(B) and sul2.

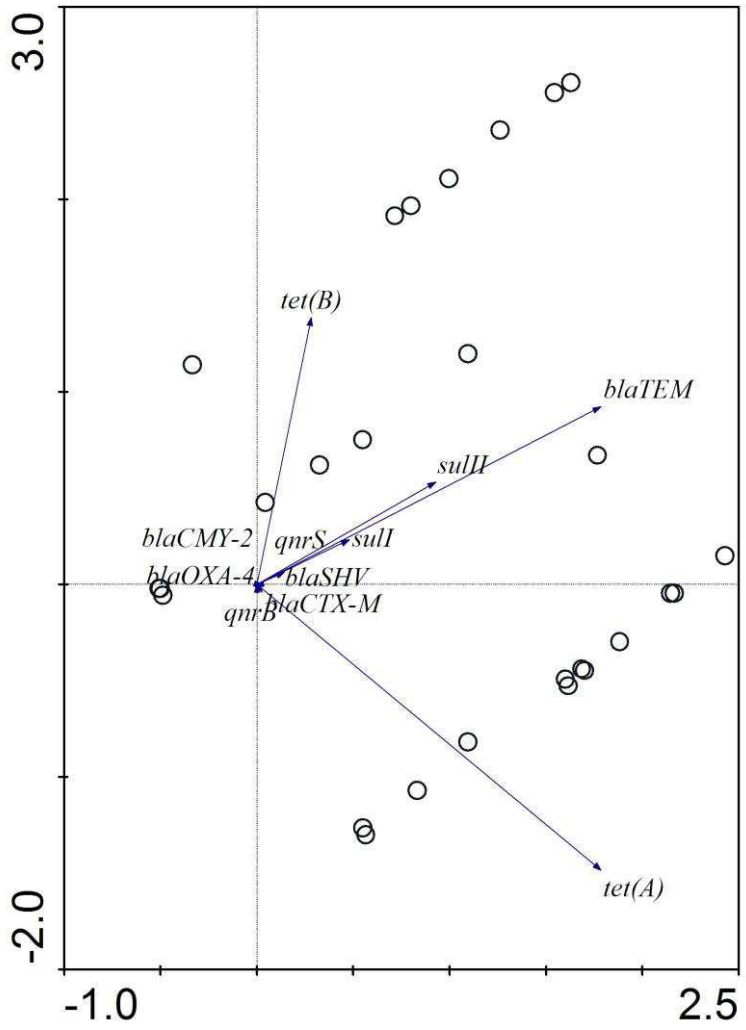


Figure III. 17 – PCA biplot of E. coli isolate scores (white circles) in terms of their ARGs profile (arrows represent resistance genes). Axes represent first (47.4%) and second principal component (23.4%)

Significant differences in PC1 scores were found among sites [ANOVA: $F(2, 411) = 15.5$, $p < 0.001$], due to a significantly higher PC1 score in seawater and feces isolates comparatively to sewage isolates (Tukey test, $p < 0.05$). On the contrary, no significant spatial differences were found for PC2 scores [ANOVA: $F(2, 411) = 0.44$, $p = 0.643$]. The significant differences among sites are biased due to the low number of scores in sewage. No significant differences were found across phylogenetic groups for PC1 [ANOVA: $F(3, 410) = 1.88$, $p = 0.133$]and PC2, [ANOVA: $F(3, 410) = 1.58$, $p = 0.193$].

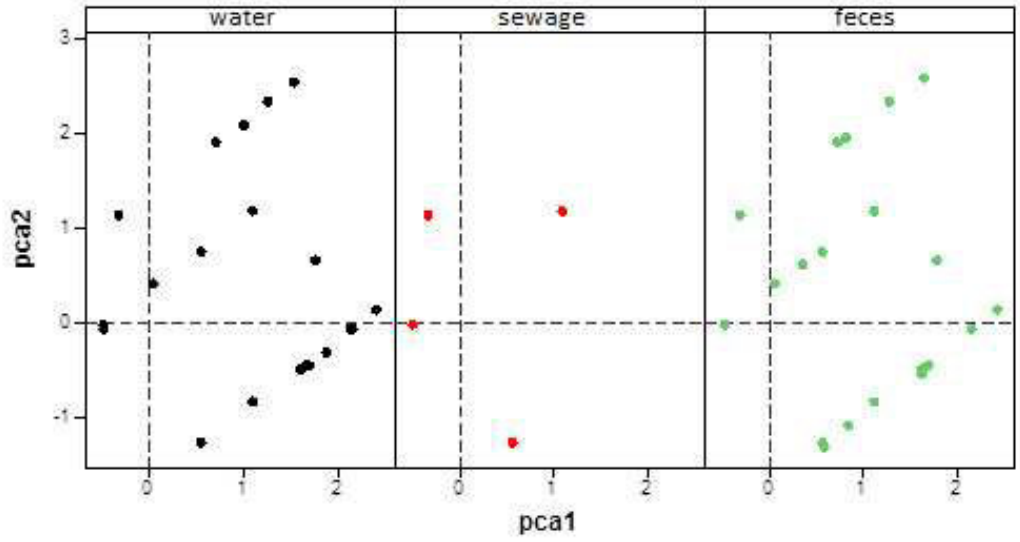


Figure III. 18 – PCA biplot of E. coli isolate scores (circles) in terms of their ARGs profile (arrows were removed to facilitate visualization) depicted according to site of origin. Axes represent first (47.4%) and second principal component (23.4%).

The variation of PC1 and PC2 scores relatively to the number of classes that isolates were resistant to were analyzed (Figure III.19 and 20). Significant differences were found in terms of PC1 [ANOVA: $F(6, 407) = 28.0$, $p < 0.001$] and PC2 [ANOVA: $F(6, 407) = 9.64$, $p < 0.001$] scores, confirming an AR gradient, with less resistant isolates consistently displaying lower PC1 and 2 scores (i.e., being located at the left of the biplot) and more resistant isolates displaying higher PC1 and 2scores (i.e. most isolates resistant to six classes of antibiotics being placed at the upper right quadrant of the biplot).

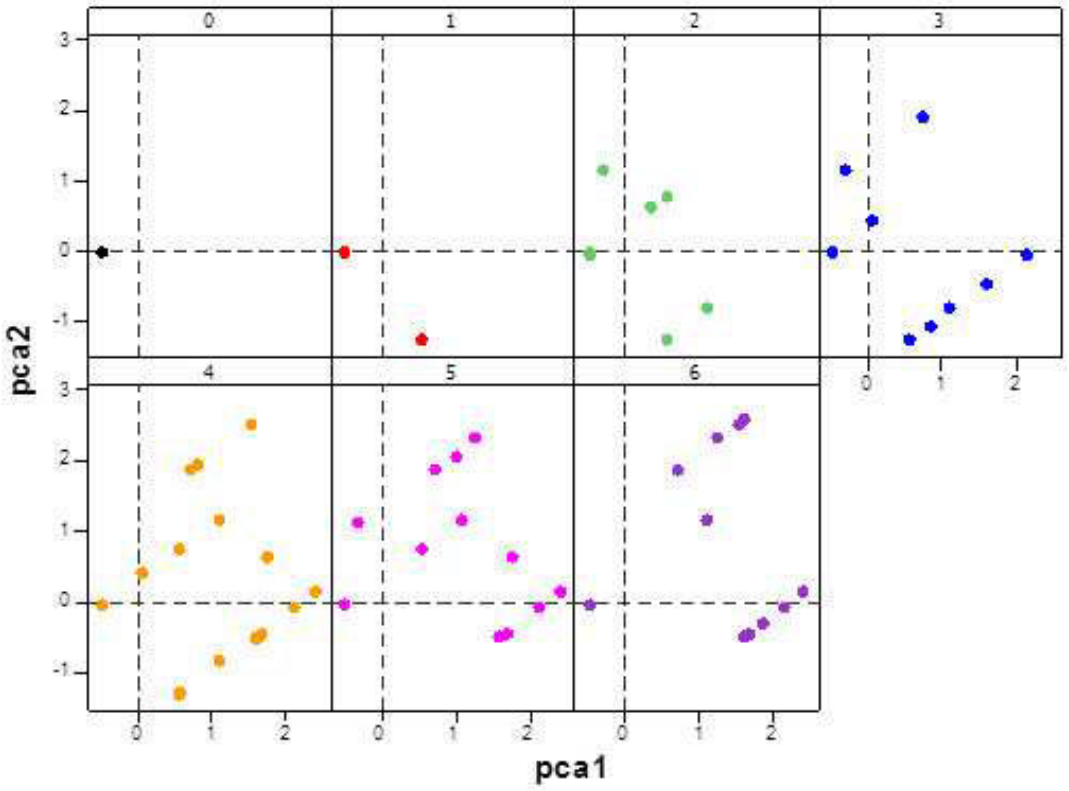


Figure III. 19 – PCA biplot of E. coli isolate scores (circles) in terms of their ARGs profile (arrows were removed to facilitate visualization). Colors represent the number of antibiotic classes that isolates are resistant to (0-6). Axes represent first (47.4%) and second principal component (23.4%)

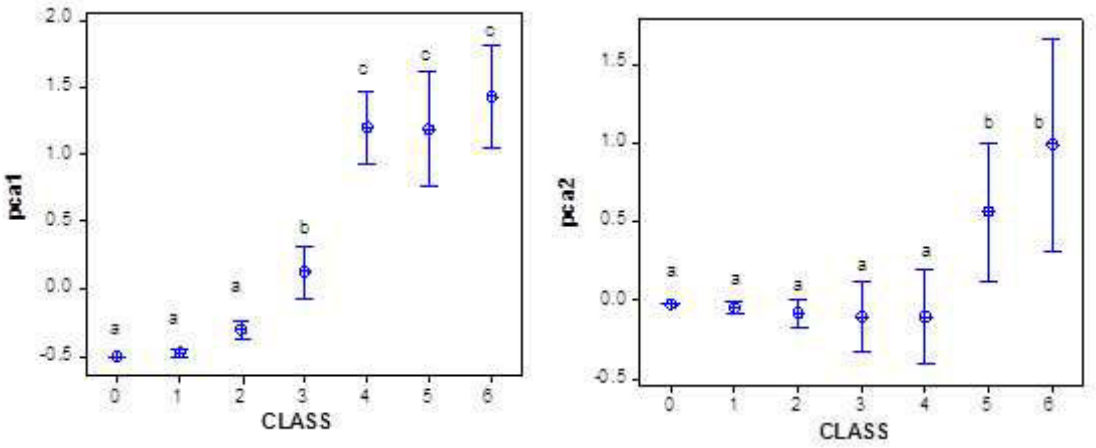


Figure III. 20 – ARGs profiles of E. coli isolates: variation of first (PC1, left) and second principal (PC2, right) components extracted from PCA across resistance classes (number of antibiotic classes that isolates were resistant to). Lowercase letters represent statistically significant differences among resistance classes (Tukey test, $p < 0.05$).

The diagram from figure III.21 explores the AR profiles obtained from PCA analysis according with the isolates genotype. In general it is possible to see that the distribution of the genotypes is influenced by the AR profile which is likely to happen once the selection of isolates for the PCR screening was preformed according with the phenotype results. In one-gene genotypes it is easy to understand this distribution: B genotypes are observed in the negative region of the PC2 axis, corresponding to a phenotype of resistance to penicillins and cephalosporins conferred by bla_{TEM} genes.

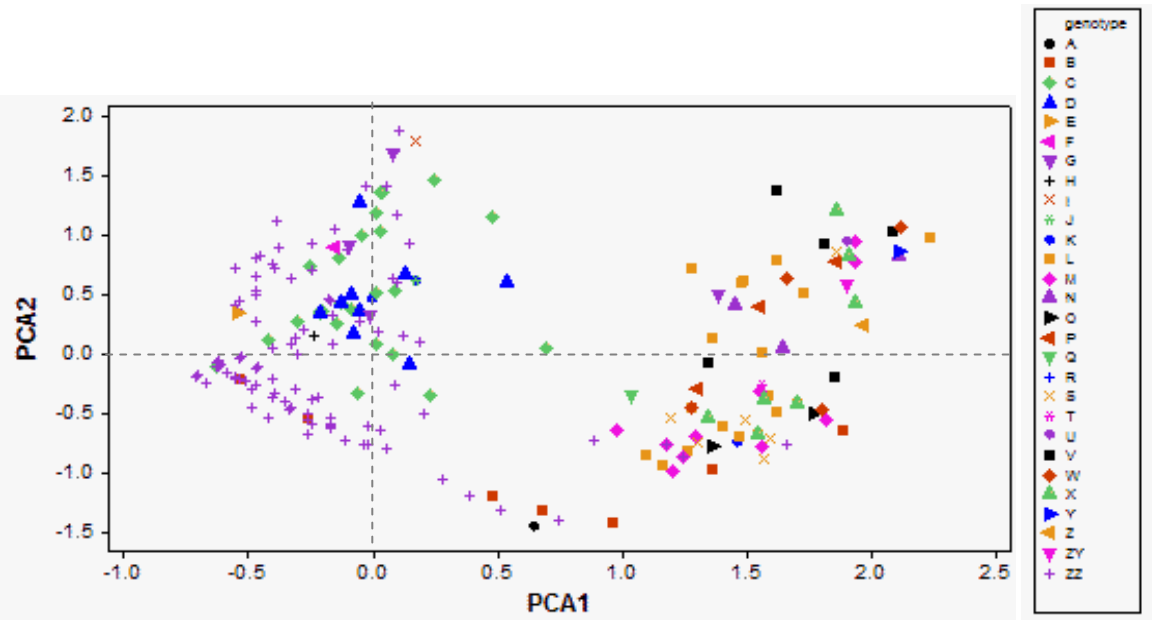


Figure III. 21. – PCA biplot of E. coli isolate scores in terms of their AR profile (arrows were removed to facilitate visualization). Symbols represent the isolate genotypes listed in table III.5. Axes represent first (44.1%) and second principal component (9.7%).

Table III.5 – Correspondence between the letters from figure III. 21. and the genotypes.

Letter	Genotype
A	bla_{CMY-2}
B	bla_{TEM}
C	tet(A)
D	tet(B)
E	qnrB
F	qnrS
G	sul2
H	sul1 + sul2
I	tet(A) + qnrB
J	tet(A) + sul1
K	tet(A) + sul2
L	bla_{TEM} + tet(A)
M	bla_{TEM} + tet(B)
N	bla_{TEM} + sul2

Letter	Genotype
O	bla_{TEM} + tet(A) + tet(B)
P	bla_{TEM} + tet(A) + qnrS
Q	bla_{TEM} + tet(A) + qnrB
R	bla_{TEM} + tet(A) + sul1
S	bla_{TEM} + tet(A) + sul2
T	bla_{TEM} + tet(B) + qnrS
U	bla_{TEM} + tet(B) + sul1
V	bla_{TEM} + tet(B) + sul2
X	bla_{TEM} + tet(A) + sul1 + sul2
W	bla_{TEM} + tet(B) + su11 + sul2
Y	bla_{TEM} + bla_{SHV} + tet(A) + sul2
Z	bla_{TEM} + $bla_{CTX-M-1}$ + tet(A) + qnrS
ZY	bla_{TEM} + tet(B) + qnrS + sul1 + sul2
ZZ	no positive PCR results for the genes tested

Focusing on the scores corresponding to isolates with no PCR positive results for the genes screened (ZZ) some of them are in regions of the plot corresponding to a certain resistance phenotype as expected, once only 40.3% of the resistant isolates had at least one of the resistance genes tested.

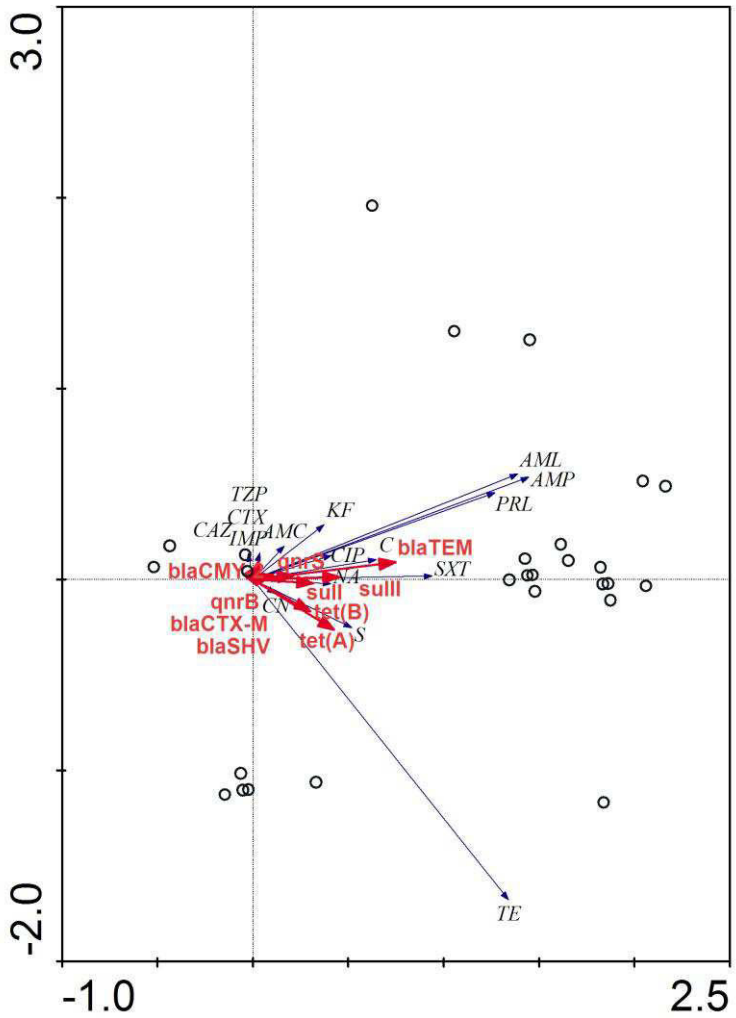


Figure III. 22 - RDA triplot of the E.coli isolates AR scores and the resistance genes screened. Blue arrows represent antibiotics and red arrows represent resistance genes. Axes represent first (35.4%) and second principal component (5.8%).

RDA (Figure III.22.) was used to determine the relationship between the AR profile and the resistance genes, demonstrating that the combination of resistance genes explains almost half (45.7%) of the AR profile. The RDA model was significant (Monte-Carlo permutation test, $p < 0.001$).

IV. DISCUSSION

The purpose of this study was to investigate the prevalence of AR in E. coli from three sources in the Berlenga Island: seagull feces, human-derived sewage and water from the beach. This archipelago has been classified as Biosphere Reserve but fecal contamination has been recurrently detected in the beach water (Araújo 2012; Araújo S. 2013). The main origin of this contamination was suggested to be seagull feces (Araújo 2012; Araújo S. 2013). This study also aims to compare AR patterns retrieved from the three sources, assess risks associated to AR in this environment and identify AR phenotypes and/or genotypes that may contribute to discriminate the sources of fecal pollution.

The affiliation of the E. coli isolates with the four principal phylogenetic groups showed that in the three sources more than 70% of the isolates belonged to group A or B1. According to the literature, while groups B2 and D usually belong to extraintestinal pathogenic strains (Picard, Garcia et al. 1999), A and B1 phylogroups belong to commensal strains (Bingen, Picard et al. 1998) which justifies the results given the probable fecal origin of the isolates.

Despite these results it is necessary to take into account that B1 strains are able to survive for long periods in the environment, especially in aquatic environments, and so they are not good host indicators (Walk, Alm et al. 2007).

In other studies it is established an association between strains of the group B2 and human-derived contamination (Carlos, Pires et al. 2010) and this may justify the high percentage of isolates from B2 phylogenetic group in the human sewage. Gull feces also contain a small percentage of B2 strains which may reflect the close contact of these wild birds with the human environment because though gulls nest in Berlengas, they frequently fly to Peniche coast where they may eat human food disposals.

Despite the appearance of B2 isolates in feces and sewage no B2 isolate was collected in water. This may reflect the low ability of B2 isolates to adapt to the seawater environment.

In terms of AR we found high levels of resistance in this preserved area with 92.2% of the Berlenga Island isolates showing resistance to at least one antibiotic of the sixteen antibiotics tested. Also multiresistance rates were around 30% in all sources.

Comparing the AR levels of Berlenga gull feces E. coli isolates between 2007, with a 53 isolates collection, (Radhouani, Poeta et al. 2009) and the present study it is possible to detect an increase in the resistance to the combination amoxicillin/clavulanic acid and in streptomycin: for the combination amoxicillin/ clavulanic acid the percentage of resistant isolates was 1.9% and in 2011, 10.6%; 32.1% of the 2007 isolates were resistant to streptomycin and in 2011, 83.2%. This raise may be related to the antibiotic (mis)use in clinics, veterinary and agriculture and consequently, thanks to the easily dissemination among bacteria, it affects also wild birds that indirectly get in contact with antibiotics.

Resistance to penicillin, aminoglycosides (streptomycin) and tetracyclines was of common occurrence in all sources, except for the less used combinations amoxicillin/clavulanic acid and piperacillin/tazobactam and gentamicin which is consistent with other studies in water environments (Dolejska, Bierosova et al. 2009; Alouache, Kada et al. 2012; Matyar 2012; Pereira, Santos et al. 2013) and in gull feces (Dolejska, Bierosova et al. 2009; Radhouani, Poeta et al. 2009; Martiny, Martiny et al. 2011). These antibiotics are older and of frequent use in human and veterinary medicine which results in highly disseminated resistance.

Resistance to gentamycin was low in all sources what can be explained by the its infrequent use and the later introduction in the clinics and veterinary medicine (van Hoek, Mevius et al. 2011).

The levels of resistance to the antibiotics tested are very similar between water and feces. In general gull feces isolates have a higher level of resistance to most antibiotics than isolates from the sewage including multiresistance levels. This suggests that the selective pressures in the environment (that can be others than the presence of antibiotic) may contribute to the high levels of AR in environmental strains consisting in a risk to human health. These levels of resistance in seagulls are in accordance to what has been described in literature (Radhouani, Poeta et al. 2009; Bonnedahl, Drobni et al. 2010; Literak, Dolejska et al. 2010) in which wild birds are described not only as a reservoir but also as a vector for AR dissemination once they live close to the human environment, acquiring AR by feeding themselves of human disposals. On the other hand seagulls are migratory birds, accumulating and transferring ARGs between distant geographic zones.

Low resistance levels were detected for recent antibiotics used mainly in humans and in clinics: imipenem, cefotaxime, ceftazidime, ciprofloxacin and the combination

piperacillin/tazobactam. Though in general the resistance levels to these antibiotics were low, in the sewage isolates they were higher than in water and feces. This difference is accentuated in the imipenem once this antibiotic, as all the carbapenems, is only used in hospitals in Portugal as a last-resort solution (Henriques, Araujo et al. 2012).

It is important to highlight that although the levels of resistance to recent antibiotics are in general low and more frequent in sewage, resistance also occurs in the environment isolates (from water and gull feces) suggesting the beginning of the dissemination in this environment.

The most common phenotypes are in agreement with the antibiotics that had higher prevalence: phenotypes combining aminoglycosides, penicillins and tetracyclines. Analyzing the phenotypes from the different sources it was possible to establish an association between the AR profile of gull feces and water: (1) the most common phenotype in water (apart from streptomycin), the cefalotin and streptomycin phenotype, is not represented in the sewage but it is as well one of the most prevalent in feces; (2) also the detection in water of AR phenotypes exclusive from seagull feces isolates was more frequent (10 phenotypes) than the detection of AR phenotypes exclusive from sewage isolates (2 phenotypes) .

In general the results from this study confirm the consequences of different selective pressures in the isolates AR pattern: isolates from gull feces did not have a significant resistance to clinic antibiotics and human gut isolates (from the human sewage) showed a higher resistance to those antibiotics. These results also support the significant contribution of gull feces to the water fecal contamination due to the similarity of the resistance levels of these two sources and in fact the statistical analyses showed a significant difference between water and sewage, but not between water and feces in accordance with the stated in previous studies (Araújo 2012; Araújo S. 2013).

The distribution of antimicrobial resistance among E. coli phylogenetic groups was also analyzed. It has been reported in some studies a trend for lower antimicrobial resistance frequency in group B2 (Skurnik et al, 2005; Radhouani et al, 2009) which is in agreement with the results presented here: only half of the B2 isolates had resistance to at least one of the antibiotics tested and B2 isolates were the only ones that did not show resistance to more than 4 classes of antibiotics. Groups A and D were the ones more frequently resistant to the antibiotics tested as described in other studies (Pereira, Santos et

al. 2013). The differences mentioned may not be significant once the number of B2 isolates was much lower than the other group's isolates.

Phenotypes which included imipenem resistance, an antibiotic used only in humans as previously mentioned, are more often found in B1 isolates as reported in literature for environments with human impact (Sabate, Prats et al. 2008; Mataseje, Neumann et al. 2009; Pereira, Santos et al. 2013).

The genes tested in this study were chosen among the most prevalent and transferable AR genetic determinants in E. coli considering both clinical and environmental relevance.

Genes bla_{TEM}, tet(A), tet(B), sul1and sul2 were the more frequently amplified in agreement with other studies in E. coli isolates (Hamelin, Bruant et al. 2007; Dolejska, Bierosova et al. 2009; Pereira, Santos et al. 2013). The bla_{TEM} genes of isolates with resistance to penicillins and cephalosporins were sequenced searching for ESBL encoding genes. However, all bla_{TEM} genes were identified as bla_{TEM-1}, encoding a non-ESBL enzyme. bla_{TEM-1} codes for TEM-1 β-lactamase the most common β-lactamase in Gram-negative bacteria. More than 90% of ampicillin resistance in E. coli is due to TEM-1 production (Cooksey, Swenson et al. 1990; van Hoek, Mevius et al. 2011; Korzeniewska and Harnisz 2012).

bla_{SHV} gene was detected only in a water isolate with resistance to the penicillins tested (except TZP) and to all the cephalosporins tested (ESBL phenotype). The gene was sequenced and identified as being the ESBL-encoding bla_{SHV-12} found in isolates from aquatic environments in other reports (Zurfluh, Hachler et al. 2013) and highly disseminated among clinical and environmental isolates in Spain and Portugal (Coque, Baquero et al. 2008; Radhouani, Igrejas et al. 2013).

The 3rd generation cephalosporins-resistant isolates were further tested for the presence of ESBL-encoding genes. The bla_{CTX-M} gene was detected in one water isolate. The sequenced fragment had 100% identity with $bla_{CTX-M-1}$ that is widely disseminated in Europe (Canton, Gonzalez-Alba et al. 2012) being frequently described in clinics (Eckert, Gautier et al. 2006) and in terrestrial and aquatic environments (Tacao, Correia et al. 2012; Zurfluh, Hachler et al. 2013) including seawater (Alouache, Kada et al. 2012). This gene

was also detected in gull's fecal droppings in south France (Bonnedahl, Drobni et al. 2009) and in 2008 in Berlenga (Poeta, Radhouani et al. 2008). In the present study the gene was found to occur between the insertion sequence ISEcp1 and Orf477 as previously described in literature for this ESBL gene once CTX-M genes have a conserved genomic context (Saladin, Cao et al. 2002; Poeta, Radhouani et al. 2008; Tacao, Correia et al. 2012). The literature confirms (Eckert, Gautier et al. 2006) the predominant role of ISEcp1 in the mobilization of bla_{CTX-M} genes of the CTX-M-1 cluster.

An isolate from seagull feces carried a bla_{CMY-2} gene, with the reported ISEcp1 downstream (Kang, Besser et al. 2006),. This gene has been described as encoding the most prevalent of the plasmid mediated AmpC enzymes in Enterobacteriaceae (Su, Chen et al. 2006). AmpC enzymes provide a broader spectrum of resistance than ESBLs and aren't blocked by commercially available inhibitors. These enzymes emerge firstly by induced over expression of the AmpC chromosome gene in some bacteria and with the increasing use of antibiotics it appeared in plasmids disseminating through bacteria (Philippon, Arlet et al. 2002; Jacoby, Gacharna et al. 2009). So the appearance of a plasmid mediated AmpC gene in gull feces demonstrates that they acquire resistance genes by direct or indirect contact with human environment.

The resistance phenotype to imipenem is not justified by the presence of the β-lactamases-encoding genes tested. The phenotype may be due to other resistance mechanisms like membrane impermeabilization (Little, Qin et al. 2012).

Only half (approximately) of water and feces isolates with resistance to tetracycline had positive results for tet(A) and/or (B). Even lower was the percentage of the tet genes screened in sewage tetracycline resistant isolates (~33%). There are a great number of tetracycline resistance genes previously described (Henriques, Fonseca et al. 2008; van Hoek, Mevius et al. 2011) and the resistance of the non-positive PCR isolates for tet(A) and/or (B) may be explained be other resistance genes, not screened in this study, for efflux pumps as well as other resistance mechanisms.

tet(A) genes had similar percentages among water and feces – around 69% - and half of that percentage in the sewage. Studies of AR in gull feces and water in Czech Republic detected an higher percentage of tet(A) in gull feces than in water (Dolejska, Bierosova et al. 2009). These contradictory results may be justified by the fecal pollution occurring in Berlenga beach water mainly due to the gull feces and not occurring in the Czech pond.

To all sources sul2 genes isolates were predominantly amplified than sul1 genes as already described in water environments (Hu, Shi et al. 2008; Pereira, Santos et al. 2013) and in gull feces in Czech Republic (Dolejska, Bierosova et al. 2009) and so it can't be established a relation of source and receiver between seawater and gull feces once high levels of the gene in both environments are common.

Plasmid mediated quinolone resistance was conferred by qnrB19 and qnrS1 (all qnr genes were sequenced). qnrB and S are reported in literature as the most prevalent plasmid-mediated fluoroquinolone resistance genes. A237 is an ESBL producer isolate (with $bla_{CTX\text{-}M\text{-}1}$) and also has a qnrS1 gene. A high frequency of resistance to quinolones in ESBL-producing bacteria has been previously reported (Lautenbach, Strom et al. 2001; Lavilla, Gonzalez-Lopez et al. 2008). Plasmids carrying qnr genes frequently encode ESBLs disseminating both genes among different enterobacterial species. (Lautenbach, Strom et al. 2001; Jacoby 2005; Coque, Baquero et al. 2008; Lavilla, Gonzalez-Lopez et al. 2008).

Beyond the similar percentage of tet(A) genes among water and feces and the occurrence of qnrB19 only in water and feces it is possible to find other evident similarities among the genes found in water and feces: both sources have the same number of different genotypes (n=19), higher than in the sewage isolates (n=8), and these two sources had in common seven genotypes while water and sewage had in common only one genotype. These results further support the hypothesis that gull feces constitute the major contribution to the fecal pollution detected in Berlenga beach water.

Significant differences were found among water and sewage in terms of ARGs profile but these results are biased due to the low number of scores in sewage once there are a must lower number of isolates with positive PCR results for the genes tested than the number of water and feces isolates with positive PCR results.

Our findings show a high prevalence of AR among E. coli from the Berlenga beach seawater and gull feces. Particularly meaningful are the high prevalence of multiresistance (up to 30%) and the occurrence of resistance to last-resort antibiotics, like imipenem indicating an elevated risk to human health. Significant differences observed between the sewage and the other two sources (water and feces) in terms of AR profiles suggests the

gull feces as major source of fecal contamination as determined in previous studies (Araújo 2012; Araújo S. 2013).

Resistance is becoming transversal to all environments especially with the contribution of vectors like gulls that our results, in accordance with other reports, allow to classify as reservoirs of ARGs and antibiotic-resistant bacteria. Gulls don't directly contact with antibiotics, but being omnivorous often search for food in agricultural and urban areas and in communal garbage dumps and sewage water treatment plants becoming carriers of resistant bacteria from domestic animal and human source. Transporting these resistance genes through long distances they affect drinking and recreational water quality (Literak, Dolejska et al. 2010; Araújo S. 2013)

V. CONCLUSIONS

Nowadays, AR is a public health problem as a consequence of dissemination of resistance genes facilitated by HGT in bacteria. AR is found not only in clinical isolates but also in isolates from natural environments. Humans contribute to the selection of resistant microorganisms by misuse of the antibiotics, not only in clinics, but also in veterinary and agriculture and by discharging the wastes from these segments into the environment. New resistance mechanisms may emerge in the environment and these new mechanisms can be introduced in clinics. From the current study the following main conclusions were drawn:

1. Despite the fact of being a natural reserve, the levels of AR were high in Berlenga Island demonstrating that the dissemination of AR may affect even a protected area like Berlengas.
2. Different contributions to AR by different E. coli phylogenetic groups were confirmed in this investigation.
3. This study demonstrates that fecal pollution associated to gull feces, though generally considered less dangerous than human fecal pollution, may also constitute a risk to public health and contribute to the dissemination of AR.
4. Resistance to last-resort antibiotics used preferentially on humans (imipenem, for instance) was found mainly in human-derived isolates but it was also identified in isolates from seagull feces and water, suggesting their dissemination in the environment, which represents a serious concern.
5. E. coli isolates from human and seagull feces presented different levels of AR. Resistance genotypes and phenotypes of isolates retrieved from water and seagull feces were closely related supporting previous results which indicated that seagulls were the main origin of fecal pollution in the Berlenga Beach.
6. Some genotypes and phenotypes were identified as potential markers of seagulls fecal pollution. The use of these markers must be further investigated.

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VII. APPENDICES

Appendix A - DNA Molecular weight marker

Table A.1. - Molecular weight marker used

	GeneRuler™ DNA Ladder Mix
Fragments Size (pb)	10000
	8000
	6000
	5000
	4000
	3500
	3000
	2500
	2000
	1500
	1200
	1031
	900
	800
	700
	600
	500
	400
	300
	200
	100

Appendix B – Sequences annotation

B.1. $bla_{CTX-M-1}$

AGTCTAATTCTTCGTGAAATAGTGATTTTTGAAGCTAATAAAAAACACACGTGGAATT
TAGGTTAGACTATAAATAGAAAAAGGCGTTTTGACAGACTATTCATGTTGTTGTTAATT
CGTCTCTTCCAGAATAAGGAATCCCATGGTTAAAAAATCACTGCGTCAGTTCACGCTG
ATGGCGACGGCAACCGTCACGCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGG
CGGACGTACAGCAAAAACTTGCCGAATTAGAGCGGCAGTCGGGAGGAAGACTGGGTG
TGGCATTGATTAACACAGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTT
GCGATGTGCAGCACCAGTAAAGTGATGGCCGTGGCCGCGGTGCTGAAGAAAAGTGAA
AGCGAACCGAATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACTTGGTTAACT
ATAATCCGATTGCGGAAAAGCACGTCGATGGGACGATGTCACTGGCTGAGCTTAGCGC
GGCCGCGCTACAGTACAGCGATAACGTGGCGATGAATAAGCTGATTTCTCACGTTGGC
GGCCCGGCTAGCGTCACCGCGTTCGCCCGACAGCTGGGAGACGAAACGTTCCGTCTCG
ACCGTACCGAGCCGACGTTAAACACCGCCATTCCGGGCGATCCGCGTGATACCACTTC
ACCTCGGGCAATGGCGCAAACTCTGCGTAATCTGACGCTGGGTAAAGCATTGGGTGAC
AGCCAACGGGCGCAGCTGGTGACATGGATGAAAGGCAATACCACCGGTGCAGCGAGC
ATTCAGGCTGGACTGCCTGCTTCCTGGGTTGTGGGGGATAAAACCGGCAGCGGTGACT
ATGGCACCACCAACGATATCGCGGTGATCTGGCCAAAAGATCGTGCGCCGCTGATTCT
GGTCACTTACTTCACCCAGCCTCAACCTAAGGCAGAAAGCCGTCGCGATGTATTAGCG
TCGGCGGCTAAAATCGTCACCAACGGTTTGTAATAGCGGAAACGGAATGGGGAAACT
CATTCCGTTTTTGTTTATCGCCTTAGACGGCAAAAGCGCTGTCGCCCACCTGCGCTTGC
GCATACCAGGCCATAAGCTCCGTGGTTCCTGGTTCTCCTTCCGCTGGCGCCCAGTGCGC
ATAGTCATCGGCAGCC

Xxxxxxxxxx → *$bla_{CTX-M-1}$*

Xxxxxxxxxx → Right inverted repeat (RIR)

Xxxxxxxxxx → region with 32 pb

Xxxxxxxxxx → region with 48 pb

Xxxxxxxxxx → *orf 477*

B.2. bla_{CMY-2}

TGCTGAAAACTATATCAAAGAAGCCAAATACGACATGGCGGTGGGTCATCTCTTGCTA
AAGTCATTTTGGGCGAATGAAGCCGTGTTTCAAATGATGATGCTTTCATATAACCTATT
TTTGTTGTTCAAGTTTGATTCCTTGGACTCTTCAGAATACAGACAGCAAATAAAGACCT
TTCGTTTGAAGTATGTATTTCTTGCAGCAAAAATAATCAAAACCGCAAGATATGTAAT
CATGAAGTTGTCGGAAAACTATCCGTACAAGGGAGTGTATGAAAAATGTCTGGTATAA
TAAGAATATCATCAATAAAATTGAGTGTTGCTCTGTGGATAACTTGCAGAGTTTATTAA
GTATCATTGCAGCAAAGATGAAATCAATGATTTATCAAAAATGATTGAAAGGTGGTTG
TAAATAATGTTACAATGTGTGAGAAGCAGTCTAAATTCTTCGTGAAATAGTGATTTTTG
AAGCTAATAAAAAACACACGTGGAATTTAGGAAAAACTTATATCTGCTGCTAAATTTA
ACCGTTTGTCAACACGGTGCAAATCAAACACACTGATTGCGTCTGACGGGCCCGGACA
CCTTTTTGCTTTTAATTACGGAACTGATTTCATGATGAAAAAATCGTTATGCTGCGCTC
TGCTGCTGACAGCCTCTTTCTCCACATTTGCTGCCGCAAAAACAGAACAACAGATTGC
CGATATCGTTAATCGCACCATCACCCCGTTGATGCAGGAGCAGGCTATTCCGGGTATG
GCCGTTGCCGTTATCTACCAGGGAAAACCCTATTATTTCACCTGGGGTAAAGCCGATA
TCGCCAATAACCACCCAGTCACGCAGCAAACGCTGTTTGAGCTAGGATCGGTTAGTAA
GACGTTTAACGGCGTGTTGGGCGGCGATGCTATCGCCCGCGGCGAAATTAAGCTCAGC
GATCCGGTCACGAAATACTGGCCAGAACTGACAGCAAACAGTGGCAGGGTATCCGCC
TGCTGCACTTAGCCACCTATACGGCAG

Xxxxxxxxxx → *bla_{CMy-2}*

Xxxxxxxxxx → Right inverted repeat (RIR)

Xxxxxxxxxx → region with 182 pb

Xxxxxxxxxx → region with 116 pb