Universidade de Aveiro
2021

Susana Manso Araújo

Ocorrência e disseminação de resistência a antibióticos no ambiente: origem dos genes de resistência e papel da atividade humana

Emergence and dissemination of antibiotic resistance in the environment: origin of resistance genes and role of human activities

Universidade de Aveiro
2021

Susana Manso Araújo

Ocorrência e disseminação de resistência a antibióticos no ambiente: origem dos genes de resistência e papel da atividade humana

Emergence and dissemination of antibiotic resistance in the environment: origin of resistance genes and role of human activities

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia e Ecologia das Alterações Globais, realizada sob a orientação científica da Doutora Isabel da Silva Henriques, Professora Auxiliar do Departamento das Ciências da Vida da Universidade de Coimbra e do Doutor Artur Jorge da Costa Peixoto Alves, Professor Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro.

Apoio financeiro da FCT e do FEDER através do programa COMPETE2020 no âmbito do projeto de investigação StARE.

Bolsas com referência:
WaterJPI/0002/2013
UID/AMB/ 50017-POCI-01-0145-FEDER-007638
UIDP/50017/2020+UIDB/50017/2020

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

Bolsa de Doutoramento:
SFRH/BD/52573/2014

(...), turn up the volume of your own intuition and turn down the *noise* of the world. Your lack of confidence keeps your eyes up watching everyone else but your biggest success comes when you listen to and trust your gut.

Jenna Kutcher

o júri

presidente

Doutor Rui Luís Andrade Aguiar
Professor Catedrático da Universidade de Aveiro

vogais

Doutora Maria José Félix Saavedra Môcho
Professora Associada com Agregação da Universidade de Trás-os-Montes e Alto Douro

Doutor Jorge Andrés Olivares Pacheco
Professor Associado da Pontificia Universidad Católica de Valparaíso

Doutora Gabriela Conceição Duarte Jorge da Silva
Professora Associada da Universidade de Coimbra

Doutora Sónia Alexandra Leite Velho Mendo Barroso
Professora Auxiliar com Agregação da Universidade de Aveiro

Doutora Isabel da Silva Henriques
Professora Auxiliar da Universidade de Coimbra

agradecimentos

Estes últimos anos fizeram-me voltar para dentro e pôr a vida em perspectiva, mas se alguma coisa não se alterou foi o meu agradecimento profundo à Dra. Isabel Henriques. Isabel, tenho-te uma estima e admiração enorme. Sem o teu apoio incansável, o desenlace deste trabalho não teria sido vitorioso. Obrigada!
Ao Dr. Artur Alves, pela ajuda na orientação desta tese, o meu muito obrigada.
Ao professor António Correia, apesar de já não estar entre nós, por ter ajudado a despertar o bichinho pela Microbiologia e por aceitar a minha integração na sua equipa, o meu eterno agradecimento!

À Juliana, minha mentora e minha amiga, que me transmitiu os ensinamentos básicos da investigação e a sua exemplaridade de ética de trabalho.
À Marta T. que apesar de não o ser oficialmente, ajudou-me de forma incansável e esteve sempre presente tal qual uma orientadora.

A todos os colegas do Microlab, em especial às meninas a quem tenho o prazer de chamar de amigas: Cátia, Carina, Marta, Eliana, Nádia, Fernanda, Forough, Laura, e *pupilas* Isabel e Melissa o meu muito muito obrigada. Pela ajuda, ora diretamente no laboratório e trabalho de campo, e em produtivas discussões sobre o trabalho, ora em conversas ternas que nos enchem os ouvidos e aquecem o coração quando mais precisamos, mesmo que isso significasse convívios fugazes às 3h da manhã.
A todos os restantes colegas de departamento, sempre dispostos a uma breve saudação e prontos para cinco minutos de leves *conversas de corredor*, um muito obrigada. Esses minutos fizeram toda a diferença no meu dia a dia.
Aos colegas do Brasil, obrigada por toda a cooperação e experiência incrível no país das mangueiras.

À Ana Patrícia: sem a tua ajuda, esta tese não teria sido escrita no período de tempo em que foi. Estou-te imensamente grata!

À família, obrigada pela paciência inquieta. Nunca me deixaram permanecer num lugar de conforto.
À minha família: Bruno, obrigada pela tua impaciência quase diária, que apesar de me deixar louca, fez-me crescer, mas sobretudo pelo apoio e colo; e às minhas filhas, Francisca e Benedita, que espoletaram uma volta de 180° na minha vida e me obrigaram a olhar-me, a trabalhar-me, a voltar-me para a minha essência. Obrigada por me mostrarem do que é realmente feita a vida.

palavras-chave

Resistência a antibióticos, origem de genes de resistência, carbapenemos, bactérias ambientais, ambientes aquáticos, pressão antropogénica.

resumo

A resistência a antibióticos é uma preocupação crescente no que diz respeito à saúde pessoal e comunitária, à discrepância social no acesso à saúde e ao futuro do mundo natural. Este trabalho teve como objetivos compreender a origem dos determinantes genéticos da resistência a antibióticos (RA) em bactérias ambientais, bem como explorar o impacto da pressão antropogénica na evolução e dispersão da RA.

O papel do género *Shewanella* como origem e reservatório de genes de RA foi avaliado através da análise de uma coleção de isolados ambientais e de genomas deste género, depositados em bases de dados públicas. A presença e o contexto genético do gene que codifica para a carbapenemase OXA-48 e a presença de genes *qnrA* foram avaliados. Estes genes foram detetados em várias espécies de *Shewanella*, nalguns casos pela primeira vez, sendo específicos para algumas destas espécies. Além disso, várias variantes novas foram identificadas neste trabalho. Sequências de inserção associadas à transferência de genes foram identificadas, fundamentando a sua contribuição na dispersão destes genes para outros grupos filogenéticos.

O impacto da ação humana na disseminação da RA em compartimentos aquáticos foi abordado através da análise de vegetais consumidos crus e da água subterrânea utilizada para irrigação. Foram encontradas estirpes multirresistentes e com características de virulência, comuns aos dois ambientes, sugerindo a água de irrigação como origem da contaminação detetada em vegetais. A análise do genoma de algumas destas estirpes revelou determinantes de virulência, elementos genéticos móveis e genes de resistência, sugerindo um risco potencial para a saúde humana. Além disso, a diversidade e abundância de bactérias resistentes a carbapenemos foram avaliadas numa estação de tratamento de águas residuais, ao longo do processo que inclui um passo de desinfeção com radiação ultravioleta. O tratamento reduziu significativamente o número de bactérias, totais e resistentes a carbapenemos. Em águas não tratadas, foram detetadas estirpes de *Enterobacteriaceae* com o gene bla_{GES-5} -associado a integrões-, raramente encontrado no contexto clínico em Portugal. No efluente final foram encontradas bactérias intrinsecamente resistentes aos carbapenemos, nomeadamente *Stenotrophomonas*.

Os resultados obtidos revelam evidência adicional no que diz respeito ao papel das bactérias ambientais como progenitores dos genes de RA, tal como o papel do homem na disseminação da RA nos compartimentos aquáticos. Este conhecimento é crucial para definir estratégias de mitigação deste problema, tanto no meio ambiente como na clínica.

keywords

Antibiotic resistance, resistance genes origin, carbapenems, environmental bacteria, aquatic settings, anthropogenic pressures.

abstract

Resistance to antibiotics is a rising concern in respect to community and personal health, health-access social discrepancy and the future of the natural world. This work aimed to understand the role of environmental bacteria as the origin of the genetic determinants of antibiotic resistance (AR), as well as to explore the impact of anthropogenic pressures on the evolution and spread of AR.

Shewanella's genus role as progenitors and reservoir of AR genes was assessed through the analysis of a collection of environmental isolates and genomes of this genus deposited in public databases. The presence and the genetic context of the gene encoding for carbapenemase OXA-48 and the presence of *qnrA*-like genes was assessed. These genes were detected in several *Shewanella* species, in certain cases for the first time, being species-specific at times. Furthermore, several new variants were identified in this work. Insertion sequences associated with gene transfer were identified, suggesting its contribution to the spread of these genes to other phylogenetic groups.

The impact of human action on the spread of AR in aquatic compartments was addressed through the analysis of groundwater used for irrigation and vegetables consumed raw. Multiresistant strains with virulent characteristics were found, common to both environments, suggesting irrigation water as the source of the contamination detected in the vegetables. Genome analysis of some of these strains revealed virulence determinants, mobile genetic elements and resistance genes, suggesting a potential risk to human health. In addition, the diversity and abundance of bacteria resistant to carbapenems were evaluated in a wastewater treatment plant, throughout the process, which includes an ultraviolet radiation disinfection step. This treatment showed significant results in reducing the number of bacteria, either total and resistant to carbapenems. In untreated samples, *Enterobacteriaceae* strains were detected carrying $bla_{\text{GES-5}}$ -associated with integrons-, which is rarely found in clinical settings in Portugal. In the final effluent were found bacteria intrinsically-resistant to carbapenems, namely *Stenotrophomonas*.

The results obtained in this work reveal additional evidence regarding the role of environmental bacteria as progenitors of AR genes, as well as the role of humans in the spread of AR in aquatic compartments. This knowledge is crucial to define mitigation strategies for this problem, both in the environment and in the clinic.

TABLE OF CONTENTS

I. CONTEXTUALIZATION

The discovery of antibiotics is considered one of the most significant health-related achievements of modern times. On one hand, since their introduction in the 1940s, their clinical application circumvented a major public health problem, reducing morbidity, child mortality and, common infectious diseases deaths (Centers for Disease Control and Prevention, CDC, 2011). On the other side of the balance, a not so positive course of action has emerged: antimicrobials usage has occurred so massively over the years that led to an overloaded planet striking rapid selection of resistant strains.

Large quantities of antibiotics have not only been used in the treatment of infectious diseases in human medicine and veterinary practices but widely used as prophylactic, and growth promotion agents across other activities such as livestock production and aquaculture (Kümmerer, 2009a). According to Davies and Davies point of view, it "is not a natural process, but a man-made situation superimposed on nature" (Davies and Davies, 2010), that led to the development of generations of antibiotic-resistant microbes as a result of many years of chronic selective pressure, via antibiotic use, but especially via underuse, overuse and misuse.

Antibiotics can be considered as signaling molecules as sub-MIC concentrations can have pleiotropic effects on gene expression and metabolites production (Bernier and Surette, 2013); thus, antibiotic killing activity is concentration-dependent. While in a functional way, antibiotics can be defined as synthetic or natural organic molecules for therapeutic application, which kill or inhibit microbial growth through specific biochemical interactions (Davies and Davies, 2010). Therefore, antimicrobials can act as bactericidal or bacteriostatic agents, and although the definition of antibiotic can be extended to compounds acting on other microorganisms other than bacteria, the term is most often used with respect of antibacterial substances.

In turn, bacteria have the capability of multiplying their cells exponentially. The high rate of spontaneous mutations allied by tremendous genetic plasticity gives bacteria a clear potential of adaptation to multiple new conditions (when favorable) quickly resulting in a huge number of progeny – a classic example of Darwin's principle, "survival of the fittest". An innate characteristic of bacteria, which has evolved along with mankind's demand for improved health and the

serendipity of livestock and agriculture in the last century, is antibiotic resistance (AR).

1. ANTIBIOTIC RESISTANCE

The discovery of antibiotics led to a generalized optimism that infections could be controlled and avoided. However, the emergence of AR seems inevitable to almost every new drug, and it is recognized as a major problem in the treatment of both hospital- and community-acquired microbial infections.

Resistance to an antimicrobial compound occurs when bacteria have the capacity (resistance traits) to overcome the inhibitory and lethal effects of its therapeutic application. Even if the number of infectious diseases-related deaths are reportedly decreasing in recent years (CDC, 2011), a large range of common infections in ambulatory care, such as respiratory and urinary tract infections, sexually transmitted infections or food- and water-borne infections are becoming harder, and sometimes impossible, to treat as antibiotics become less effective (Laxminarayan, 2014). This is due to not only the recurrence of diseases once controlled but more specifically due to the emergence of infectious agents relentlessly resistant to the limited range of antimicrobial drugs we have in our days.

According to the World Health Organization (WHO), since the beginning of the century infectious diseases remain as one of the 10 leading causes of death in the world (WHO, 2018). In 2015, solely considering the European Union and European Economic Area (EU/EEA), estimations were that more than 33,000 people die each year from antibiotic-resistant infections and that the growing health burden of these infections was similar to that of influenza, tuberculosis, and HIV combined (Cassini et al., 2019).

This outcome is a result of multiple factors contributing to the emergence of AR: the natural background of AR in bacteria, the abusive use of antibiotics in human and veterinary medicine, inadequate prescription (broad-spectrum antibiotics used too often or narrow-spectrum antibiotics used incorrectly) and self-medication, traveling across continents (Yong et al., 2009), food products

transportation (Faour-Klingbeil et al., 2016), antibiotics use in food animal production (Hoelzer et al., 2017), waste from antibiotics manufacturing (Larsson, 2014), water pollution (Tacão et al., 2012) and contaminated sludge used as fertilizer (Chen et al., 2016), just to name the most credited in general. Additionally, while in the last decade the demand for antibiotic-free meat is increasing in developed countries, in underdeveloped countries the growth in consumers' demand is hoarding the market for animal products, thus forcing the implementation of production practices heavily dependent on antibiotics (Topp et al., 2017). Regarding Europe, in 2006 the use of antibiotics for animal growth boost was banished in the European Union (WHO – Regional Committee for Europe, 2011), and more recently in 2018, the European Parliament has announced the prohibition of the preventive use of antibiotics in farming, which will come into force in 2022 (European Parliament and the Council, 2018).

In the "golden era", the development of new antibiotics was directly correlated to the discovery of resistant strains. Taking into account the antibiotic timeline i.e. the sequence of discovery and resistance development for the major classes of antibiotics as shown in Figure 1, its proved the unavoidable: as antibiotics have been discovered and introduced into clinical practice, the corresponding response of bacteria in the form of resistance to antibiotics has ensued. Moreover, in the mid-20th century AR was thought as of "low probability" and with a zenith of studies occurring geneticists considered unlikely bacterial genetic exchange to be the reason for such turnover (Davies, 1995). Only in the late of the century was acknowledged the significance of gene exchange on microbial evolution and that horizontal gene transfer (HGT) is a universal property of bacteria that occurred throughout eons (Davies and Davies, 2010).

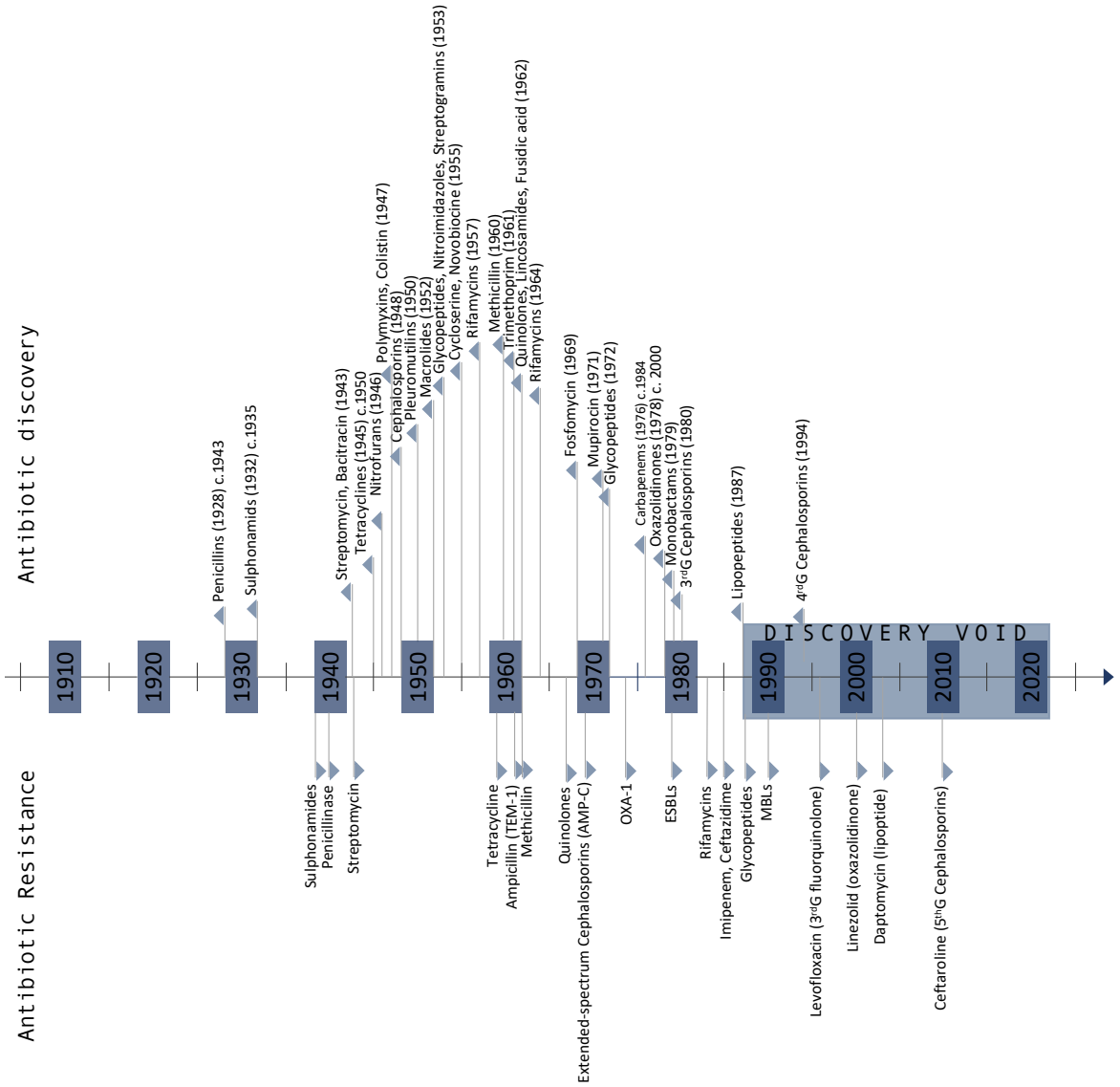


FIGURE 1. Graphical representation of antibiotics introduction and AR timeline. Adapted from (Harbarth et al., 2015; Uppsala University, 2005; Zaman et al., 2017).

Nowadays, there are still options (though limited) to treat infections, but the development and discovery of new antimicrobials is mostly idled. Given the recognized difficulty in discovering and developing new antibiotics, synergistic approaches are rising with a noteworthy advantage since they provide an opportunity to extend the life of known antibiotic drugs that have proved effective in the past 70 years (Tyers and Wright, 2019). Contrary to Gram-positive pathogens against which some new compounds have been developed (Abbas et al., 2017; Nicolas et al., 2019), recent antimicrobials to treat Gram-negative

bacteria infections are analogs of former existing drugs with improved and/or broader spectrum of activity or syncretic β-lactam/β-lactamase inhibitor combinations such as ceftazidime-avibactam and meropenem-vaborbactam and other future promising ones (Bush, 2018a; Bush and Bradford, 2019; Noval et al., 2020; Tyers and Wright, 2019). Still, one of the most current challenges remains the inhibition of class B metallo-β-lactamases (MBLs). No MBL inhibitors have proceeded into full development at final phases (II and III) of clinical protocols (Bush, 2018a; Bush and Bradford, 2019; Tyers and Wright, 2019), except for cefiderocol; which role in clinical practice is still unclear due to the findings of higher mortality rates compared to other available therapies (Noval et al., 2020). Additionally, colistin (polymyxin B), an antibiotic introduced to the antibiotic armamentarium in the mid-20th century and then rejected due to its toxicity to eukaryotic cells, has now gained a star return as a last resort option to treat serious infections caused by *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella* spp., *Escherichia coli* and other *Enterobacteriaceae* (Falagas et al., 2010). However resistance to colistin is not new, as there are several species intrinsically resistant, such as *Providencia* spp., *Neisseria* spp., *Proteus* spp., *Serratia marcescens* and *Burkholderia cepacia*, and the antibiotic is not active against Gram-positive bacteria nor anaerobes (Falagas et al., 2010).

2. MECHANISMS OF ANTIBIOTIC RESISTANCE

Antibiotic resistance is a native feature of microorganisms, as antibiotics are themselves, and most likely mankind's needs were just the trigger that urged bacteria to readapt to a constant and rapidly changing era dominated by the selective pressure of the antibiotic industrialization.

Since antibiotics introduction, every single one of them has been retackled by bacteria that somehow managed to discover a way of getting rid of them or at least cut back their presence within the bacterial cell (see Table 1). Biochemical resistance mechanisms most often used by bacteria include the following: antibiotic inactivation by enzymatic degradation or modification, target modification, altered membrane permeability, "bypass" of metabolic pathway, sequestration, and antibiotic exclusion through efflux pumps (Peterson and Kaur, 2018). Some bacteria even use a combination of several of these mechanisms. For example, tolerance to carbapenems in *P. aeruginosa* clinical isolates is a result of the interplay between decreased production of OprD porin channel, overproduction of AmpC β-lactamase activity, and activation of several efflux systems (Quale et al., 2006).

Actually, even before penicillin (penicillin G) – the first natural antibiotic discovered – was brought into extensive use in the mid-1940s, the enzyme responsible for conferring resistance to this antibiotic, a penicillinase, was identified in Gram-positive resistant staphylococci (Abraham and Chain, 1940), providing evidence that the mechanisms of resistance against many natural antibiotics were underlying in the environmental resistome (Peterson and Kaur, 2018; Wright, 2007). While for Gram-positive cocci the acquisition of penicillin-binding proteins (PBPs) with decreased affinity for common β-lactams became the more important resistance mechanism against this class of compounds, by contrast, the production of β-lactamases was and still is, the most common resistance mechanism in Gram-negative bacteria (Bush, 2018b; Drawz and Bonomo, 2010).

TABLE 1. Summary of antibiotic classes, examples of antibiotics, and respective target and mode of resistance.

Antibiotic class		Examples	Target		Mode(s) of resistance
β-lactams	Penicillins	Natural: Penicillin G, Penicillin VK Aminopenicillins: Ampicillin, Amoxicillin Penicillinase-resistant: Methicillin, Oxacillin Extended-spectrum: Ticarcillin, Carbenicillin, Piperacillin	Peptidoglycan biosynthesis	Cell wall synthesis	Hydrolysis, efflux pumps, altered target
	Cephalosporins	1st generation: Cephalothin, Cefazolin 2nd generation: Cefotetan, Cefoxitin 3rd generation: Cefotaxime, Ceftazidime 4th generation: Cefepime 5th generation: Ceftaroline			
	Carbapenems	Ertapenem, Imipenem, Meropenem, Doripenem			
	Monobactams	Aztreonam			
β-lactam inhibitors		Sulbactam, Clavulanic acid, Tazobactam, Avibactam			
Glycopeptides		Vancomycin			Reprogramming peptidoglycan biosynthesis
Others		Bacitracin			
		Polymyxins, Colistin	Cell membrane		Altered target, efflux pumps
Sulfonamides		Sulfamethoxazole	Folic acid synthesis	Nucleic acid synthesis	Efflux pumps, altered target
Pyrimidines (1DHFR inhibitor)		Trimethoprim			
Fluoroquinolones		Ciprofloxacin, Levofloxacin, Nalidixic acid	DNA replication		Acetylation, efflux pumps, altered target
Rifamycins		Rifampin	RNA transcription		ADP-ribosylation, efflux pumps, altered target
Tetracyclines		Tetracycline, Tigecycline	RNA 30S subunit	Protein synthesis	Monooxygenation, efflux pumps, altered target
Aminoglycosides		Gentamicin, Kanamycin, Tobramycin, Streptomycin			Phosphorylation, nucleotidylation, acetylation, efflux pumps, altered target
Macrolides		Erythromycin, Azithromycin	RNA 50S subunit		Hydrolysis, glycosylation, phosphorylation, efflux pumps, altered target
Phenicols		Chloramphenicol			Acetylation, efflux pumps, altered target
Oxazolidinones		Linezolid			Efflux pumps, altered target
Lincosamides		Clindamycin			Nucleotidylation, efflux pumps, altered target
Streptogramins		Dalfopristin/Quinupristin			C-O lyase (type B), acetylation (type A), efflux pumps, altered target

[1]DHFR, dihydrofolate reductase inhibitor

Several authors defend that antibiotics naturally-produced by microorganisms were designed for the producing-bacteria self-protection and competitors inhibition (Bush, 2018; D'Costa et al., 2011; Davies and Davies, 2010); while others suggest that antibiotics killing activity might be dependent of the dose and function as signaling molecules in bacterial metabolism (Bernier and Surette, 2013; Cox and Wright, 2013; Fajardo et al., 2008); by means is only natural that bacteria – producers and non-producers – would have to develop some self-protection strategies to escape from these antimicrobial compounds effects (Bush, 2018b; D'Costa et al., 2011; Davies and Davies, 2010; Peterson and Kaur, 2018). Most producer organisms, e.g. actinomycete bacteria, contain multiple mechanisms for self-protection (20–30 genetic programs on average; Katz and Baltz, 2016) for the biologically active molecules produced by themselves, and interestingly, the genetic determinants for self-protection are very often located in the same clusters as the antibiotic biosynthesis genes, and their expression co-regulated (Mak et al., 2014). Additionally, producers and non-producers co-existence is also believed to have resulted in the co-evolution of resistance mechanisms in non-producing environmental bacteria, which led to a recent interest in these bacteria in order to understand their link with the emergence of resistance in clinical pathogens (Surette and Wright, 2017). In fact, resistance genes are much more widespread in environmental non-pathogenic microbial populations than was originally believed (Bush, 2018b; Cox and Wright, 2013; D'Costa, 2006; D'Costa et al., 2011; Surette and Wright, 2017). Also, contemporary samples collected from pristine antibiotic environments further support the prevalence of AR elements in microbial resistomes not-related to modern human use (Allen et al., 2009; Bhullar et al., 2012; D'Costa et al., 2011; Henriques et al., 2012).

Bacteria can be inherently resistant, or acquire resistance elements, via HGT routes that often do not respect species or genus boundaries. Bacteria present in the natural environment are by evidence more often intrinsically resistant to antibiotics than the commensal organisms (Surette and Wright, 2017), as in the case of chromosomally-encoded MBLs expression whose presence is directly correlated with the prevalence of the producing species in a given environment (Queenan and Bush, 2007).

Intrinsic antibiotic mechanisms are usually chromosomal and comprise non-specific efflux pumps (which likely evolved as a general bacterial defense mechanism to toxins), antibiotic inactivating enzymes, or impermeability of the outer membrane in Gram-negatives (Cox and Wright, 2013; Fajardo et al., 2008). Although intrinsic mechanisms may confer low level of AR in the original host, normal commensal flora or environmental bacteria containing intrinsic mechanisms can become opportunistic pathogens in immunocompromised patients (Wright, 2007). The high-level intrinsic resistance of some Gram-negative bacteria is attributed not only to the impermeability of the outer membrane but by the synergistic mechanism between both the decreased permeability and active efflux of antibacterial agents (Cox and Wright, 2013). A very well-studied example of intrinsic resistance is the AcrAB/TolC efflux pump system in *E. coli*, which has a very broad substrate specificity and can export a broad range of antibiotics (Potter et al., 2016). Resistance to β-lactams, specifically carbapenems, in ubiquitous aquatic Gram-negative bacteria like *Stenotrophomonas maltophilia* provides another very well documented example of intrinsic resistance. This emerging pathogen co-produces two inducible chromosomally encoded β-lactamases, the L1 carbapenemase and the L2 cephalosporinase; these intrinsic resistance mechanisms along with low outer membrane permeability and multidrug resistance efflux systems gives this environmental opportunistic bacterium a clear advantage when infecting vulnerable and immunocompromised patients (Adegoke et al., 2017; Avison et al., 2001; Fajardo et al., 2008).

The acquired resistance mechanisms, instead, are generally a result of DNA mutations or HGT. HGT can occur by three main processes: conjugation between two bacterial cells, transformation of free DNA into a bacterial cell, and transduction via phage mediation. Nevertheless, plasmid-mediated conjugation is still thought to be far more prevalent in disseminating antibiotic resistance genes (ARGs) than either transformation or transduction (Von Wintersdorff et al., 2016). Plasmids are capable of autonomous replication and can carry ARGs against all major classes of antibiotics, but, indeed, can also carry a collection of other resistance genes as part of transposons, thus simultaneously conferring resistance to other compounds such as "heavy" metals (Carattoli, 2013, 2009). While it is not clear why and how resistance genes are captured or transferred from the chromosome to plasmids, the role of insertion sequences (ISs) and transposons, along with the presence of integrons, greatly aid the mobilization of resistance

genes. While integrons are not self-mobile, they can be mobilized to plasmids or phages by transposons, thus gaining the ability to move between long genetic distances to different species, genera, and even kingdoms (Carattoli, 2013). As gene transfer by conjugation can be easily tracked, evidence shows plasmids can transfer genes among unrelated bacteria depending on the host range of the plasmid, establishing their contribution to the worldwide dissemination of AR determinants both in the community and clinical environments (Carattoli, 2013). Moreover, the resistance genes found in clinic belong to the same functional families as the ones of producer organisms (Forsberg et al., 2012). Yet, the genetic context, expression, and distribution of ARGs in clinical strains are indeed different from those found in natural producers. For instance, resistance elements originally embedded in biosynthesis gene clusters are likely often found on plasmids and transposons in clinical strains. Some of the most successful plasmids are the ones that have contributed to the spread of carbapenemase genes, *bla*$_{CTX-M}$ ESBLs, and quinolone resistance genes among Gram-negative bacteria over distant geographic areas (Carattoli, 2013). A well-documented paradigm of such a phenomenon is the mobilization of chromosomal β-lactamase *ampC* genes to plasmids resulting in their worldwide dissemination (Bush and Bradford, 2019; Jacoby, 2009). Several plasmid-encoded *ampC* genes are known, such as the most commonly found worldwide encoding CMY enzymes, and other enzyme families, namely FOX, MOX, ACC, LAT, MIR, ACT, and DHA. Some of them seem to derive from chromosomal genes along with the assistance of ISs (IS*Ecp1* and IS*CR1*) as their genetic environment, likely turning them the progenitors of the plasmid-encoded enzymes (Jacoby, 2009).

2.1. Resistance to β-lactams

β-lactams are arguably the most successful antimicrobial class due to their excellent safety profile (low toxicity for the host), efficiency, and broad-spectrum of activity. These are important facts that make them constitute the most prescribed in human clinical settings and approximately 50% of all prescribed antimicrobials in veterinary settings globally (Bush and Bradford, 2016; ECDC/EFSA/EMA, 2017; European Center for Disease Prevention and Control et al., 2015; Trade and Agriculture Directorate and Committee for Agriculture, 2019). Also, according to three European Agencies (ECDC, EFSA, and EMA) joint

data, Portugal is the second-largest consumer of carbapenems in humans (either in the community and hospital) right behind Greece (ECDC/EFSA/EMA, 2017). Unfortunately, resistance to this important and efficient class of antibiotics has sprouted worldwide slightly after their introduction into medical practices.

β-lactam drugs exhibit a bactericidal effect by inhibiting peptidoglycan synthesis causing the lysis of bacterial cells. They have been cataloged into four major groups: penicillins, cephalosporins, carbapenems, and monobactams, all of which include in their structure a four-membered azetidinone ring. Combinations with β-lactamase inhibitors are used as therapeutic choices in significant clinical threats to these life-saving drugs (Bush, 2018a).

There are four primary mechanisms by which bacteria can avoid the bactericidal effects of β-lactams: (i) β-lactamase enzyme production is the most common and effective mechanism of resistance in Gram-negative bacteria (Bush, 2018b; Drawz and Bonomo, 2010); (ii) changes in the active site of PBPs can lower the affinity for β-lactam antibiotics and subsequently increase resistance to these agents, being this mechanism responsible for resistance to penicillin in Gram-positive pneumococci and methicillin resistance in staphylococci (Chambers, 1999); (iii) absence or reduced expression of outer membrane proteins (OMPs) in Gram-negative bacteria, namely *Enterobacteriaceae* (Oteo et al., 2008), *P. aeruginosa* and clinical multidrug-resistant (MDR) *A. baumannii* that exhibit resistance to carbapenems based on the loss of porins (Fajardo et al., 2008; Poirel and Nordmann, 2006; Studemeister and Quinn, 1988). Of note, the isolated disruption of OMPs is not always sufficient for producing the resistance phenotype, and typically this mechanism is found in combination with β-lactamase expression in many Gram-negatives (Beceiro et al., 2011; Livermore, 1992); (iv) overexpression of efflux pumps that are capable of exporting a wide range of substrates from the periplasm or cytoplasm to the surrounding environment; efflux pumps can occur as an acquired resistance mechanism (e.g. the most common mechanism of resistance to tetracyclines in Gram-negatives), or as an intrinsic mechanism (e.g. in the case of resistance in *P. aeruginosa* to tetracyclines and chloramphenicol, but also involved in resistance to other drugs including fluoroquinolones and β-lactams) (Li and Nikaido, 2004; Poole, 2004). Moreover, these pumps are an important determinant of multidrug resistance (i.e.

resistance to more than two classes of antibiotics) in many opportunistic Gram-negative pathogens (Fajardo et al., 2008; Li and Nikaido, 2004).

β-lactamases are able to break the β-lactam ring by hydrolyzing the amide bond so rendering these antibiotics inactive. They have been categorized, based on their amino-acid sequence into classes A to D (classification herein adopted) (Ambler, 1980), and according to substrate hydrolysis and inhibitor profiles into groups 1 through 4 (Bush et al., 1995; Bush and Jacoby, 2010). β-Lactamases of Ambler classes A, C, and D are all serine β-lactamases, whereas class B comprises MBLs (Ambler, 1980). So far, more than 2,770 unique β-lactamases have been documented (Bush, 2018b). β-lactamases are thought to be the most common resistance mechanism against β-lactams that contributes to widespread resistance among Gram-negative microorganisms (Bush and Jacoby, 2010). Resistance facilitated by β-lactamase production is either plasmid-mediated or chromosomally expressed. From a general perspective, β-lactamases can be divided into two main groups regarding their target: (i) serine β-lactamases from Ambler classes A (penicillinases and extended-spectrum β-lactamases – ESBLs), C (cephalosporinases – AmpC) and D (OXA-type ESBLs, able to hydrolyze cephalosporins), and (ii) carbapenemases which can be serine carbapenemases of molecular classes A and D (OXA carbapenemases) and MBLs from Ambler class B (Ambler, 1980; Bush and Bradford, 2019; Bush and Jacoby, 2010).

Class A serine β-lactamases comprise the extended-spectrum β-lactamases (ESBLs) family, including the CTX-M enzymes (Zhao and Hu, 2013) as well as the extended-spectrum SHV and TEM types. This family of enzymes are often encoded on plasmids and thus widely promiscuous through all sort of environments (Davies and Davies, 2010; Woodford et al., 2011). Among β-lactamases, ESBLs are among the most given of attention by the scientific and medical community over the last decades. In general, they are known for their ability to hydrolyze oxyimino-β-lactams (3rd and 4th generation cephalosporins) such as cefotaxime and ceftazidime and monobactams such as aztreonam but not capable of efficiently degrade cephamycins (2nd generation cephalosporins) as cefoxitin and cefotetan and carbapenems. Furthermore, ESBLs are generally susceptible to β-lactamase inhibitors, but there are exceptions (Drawz and Bonomo, 2010).

The first ESBLs originally derived from mutations on narrow-spectrum TEM or SHV penicillinases (TEM-1, TEM-2, and SHV-1 types), and thus gained the ability to also inactivate extended-spectrum drugs and monobactams (Bush and Bradford, 2019; Bush and Jacoby, 2010). For instance, TEM-1 was the primary cause of ampicillin resistance in *E. coli* (Beceiro et al., 2011). Unlike the TEM and SHV ESBLs, CTX-M type enzymes were acquired *de novo* by lateral gene transfer from *Kluyvera* spp. (Humeniuk et al., 2002).

Among the many ESBLs described in a variety of pathogens, CTX-M along with TEM, and SHV variants have proved to be the most successful in terms of promiscuity and dissemination across various epidemiological niches (D'Andrea et al., 2013; Livermore et al., 2007). These enzymes are frequently linked to plasmid-mediated transfer, specifically, the CTX-M enzymes, which are the most commonly isolated in many parts of the world, particularly associated with outbreaks in Europe (Livermore et al., 2007). CTX-M β-lactamases are commonly found in Gram-negative *Enterobacteriaceae* such as *E. coli* and *K. pneumoniae* but have also been found in other species of this family, including *Salmonella* spp., *Shigella* spp., *Citrobacter freundii*, *Enterobacter* spp. and *S. marcescens* as well as some non-fermentative bacteria such as *P. aeruginosa*, *S. maltophilia*, and *Acinetobacter* spp. and other Gram-negatives (*Aeromonas* spp. and *Vibrio* spp.) (Bradford, 2001; Rafael Cantón et al., 2012; Livermore et al., 2007; Paterson and Bonomo, 2005).

CTX-M expression is quite often associated with co-resistance to other compounds critically reducing response to treatment (Tacão et al., 2014). The capture of these $bla_{\text{CTX-M}}$ genes from several environmental parent *Kluyvera* species by highly mobilizable structures has been creating the so-called "epidemic resistance plasmids" often carried by MDR and virulent high-risk clones (Rafael Cantón et al., 2012; D'Andrea et al., 2013). This association of $bla_{\text{CTX-M}}$-like genes to mobilizable genetic structures that carry along other genetic determinants encoding resistance to other antibiotics greatly facilitates their mobilization by highly effective clones found widely distributed, showing a great epidemiological success (Woodford et al., 2011). The most paradigmatic examples are represented by the pandemic *E. coli* ST131 clone (phylogenetic group B2), which has greatly contributed to the global dissemination of CTX-M-15 (Rogers et al., 2011), and the case of an *E. coli* ST10 strain (phylogenetic group

A), which is a typical member of the human gut microbiota but also responsible for intestinal and extra-intestinal infections, that was associated with the dissemination of various CTX-M enzymes (CTX-M-1, -2 and -9) (Valverde et al., 2009).

The same tendency is verified in Portugal where acquired CTX-M-like ESBLs have been reported mostly in *Enterobacteriaceae* species, encoding CTX-M-15 mainly, but also CTX-M-1, -14, -27 and -32 variants, retrieved from human-impacted environments (Alves et al., 2014; Silva et al., 2018; Tacão et al., 2012), healthy individuals (Machado et al., 2004) and clinical strains (Oliveira et al., 2019); mostly $bla_{\text{CTX-M-1}}$ in wild animals (Costa et al., 2006; Garcês et al., 2019; Poeta et al., 2008) and pets (Costa et al., 2006) and $bla_{\text{CTX-M-15}}$ in *K. pneumoniae* strains from nosocomial settings (Conceição et al., 2005; Machado et al., 2006) and WWTPs (Silva et al., 2018) either. Findings in non-enterobacterial species include CTX-M-15 in nosocomial *A. baumanii* (Manageiro et al., 2012) and *Pseudomonas* sp. (Tacão et al., 2014) and CTX-M-3 in *Aeromonas hydrophila* (Tacão et al., 2014).

There are also other families of acquired (generally plasmid-mediated) ESBLs clinically important but found in much lower prevalence than CTX-M types, among which are the enzymes GES, PER, VEB, BES, BEL, SFO and TLA (Bradford, 2001; Livermore, 2008; Naas et al., 2008). Some of these are found confined geographically like PER-1, found almost exclusively in Turkey (Vahaboglu et al., 1995) and Korea (Yong et al., 2003), and PER-2 in Argentina (Bauernfeind et al., 1996), while others are becoming more widespread as VEB which has been found in southeast Asian countries (Girlich et al., 2002; Poirel et al., 1999), western Europe (Naas et al., 2006; Poirel et al., 2003b), and Africa (Ouertani et al., 2016; Potron et al., 2009).

Class D OXA-type enzymes are mostly found in non-fermenters such as *Pseudomonas* spp. and *Acinetobacter* spp., with weak effect on broad-spectrum cephalosporins and clavulanic acid (Bradford, 2001; Evans and Amyes, 2014). Several of these OXA β-lactamases, such as OXA-11, -13, -14, -16, -17, -19, and -28 variants of OXA-10, and OXA-15 mutant of OXA-2 are associated with an ESBL phenotype (Evans and Amyes, 2014; Livermore, 2008).

AmpC β-lactamases confer resistance to most cephalosporins, including broad-spectrum cephalosporins (3rd and 4th generations, when enzyme overexpression occurs) as well as cephamycins. To a lesser extent, they are also able to hydrolyze penicillins and some monobactams (Bush and Jacoby, 2010; Jacoby, 2009). Chromosomal β-lactamases are produced by many species of Gram-negative bacteria, being especially important in *Enterobacteriaceae* clinical isolates of *C. freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *S. marcescens* but also in *P. aeruginosa* species. Although generally low, their expression can be induced to higher levels (and may also be constitutively expressed) following exposure to some β-lactams (e.g. ampicillin and clavulanic acid) in these organisms (Jacoby, 2009). AmpC cephalosporinases can also be expressed from plasmids, which have become widely disseminated and thus the most frequently found ESBL in this group (Bush and Bradford, 2019). Some of these variants include CMY-1, originating from *C. freundii*; the MIR and ACT families, originating from *Enterobacter* spp.; and the DHA family, originating from *Morganella morganii* (Alvarez et al., 2004; Jacoby, 2009). High-level expression of plasmid-encoded AmpC variants is mainly due to strong promoters and high gene copy numbers (Jacoby, 2009).

2.1.1. Resistance to carbapenems

Carbapenem antibiotics are generally considered to be the last resort group of antibiotic agents to treat patients with severe bacterial infections, mostly caused by Gram-negative bacteria. The rapid emergence and widespread dissemination of carbapenem-resistant *Enterobacteriaceae* (CRE) have been sadly noticed in the last decade (Potter et al., 2016). This reality increases the epidemiological risk associated to healthcare systems due to the lack of antimicrobial therapy options, causing a severe public health crisis (Bassetti et al., 2019; van Duin et al., 2013; van Duin and Doi, 2017). In a 2013 report, CRE were listed as one of the three most urgent antimicrobial resistance threats (Centers for Disease Control and Prevention, 2013), and in 2017 the WHO listed CRE as one of the most critical priority group of pathogens to be a target for research and development, along with 3rd generation cephalosporin-resistant *Enterobacteriaceae* and carbapenem-resistant *A. baumanii* and *P. aeruginosa*

(WHO, 2017). This surge in CRE is mostly driven by the emergence and spread of carbapenemases, which are β-lactamases capable of hydrolyzing carbapenems.

Carbapenemase production is certainly the most prominent mechanism underlying carbapenem resistance in Gram-negative pathogens. Other mechanisms can act complementing carbapenemase production or function together, such as (I) overproduction of Ambler class C β-lactamases (AmpC β-lactamases) or production of ESBLs (e.g., SHV, TEM, CTX-M type β-lactamases) with OM proteins loss or alteration, (II) overproduction of certain efflux pumps, and (III) alterations in the active site of PBPs. In contrast, carbapenemase production usually results in clinically relevant levels of carbapenem resistance, but on occasion may only yield reduced susceptibility to these agents. Although CRE poses challenges with treatment in general, carbapenemase-producing *Enterobacteriaceae* is considered to be of more significant concern for both infection prevention and treatment; mainly because carbapenemase genes are carried mostly on plasmids capable of being transferred between bacterial species.

Carbapenemases belong to 3 major groups of Ambler classification: MBLs from molecular class B, those which require at least one zinc ion at the active site, and serine carbapenemases of molecular classes A and D with a serine amino acid on the active site (Ambler, 1980; Bush and Jacoby, 2010).

Among MBLs are the families VIM, IMP, GIM, and SIM, and more recently the NDM enzymes (Patel and Bonomo, 2013; Queenan and Bush, 2007); which are associated with a variety of mobile platforms, where they are frequently incorporated as gene cassettes in integrons or along with other genetic resistance traits in transmissible plasmids (Moura et al., 2009; Queenan and Bush, 2007). Since their detection, SPM-, GIM-, and SIM-families have been constrained to their countries of origin (Queenan and Bush, 2007). Contrarily, acquired VIM and IMP enzymes continue to be detected worldwide, of which IMP-1 and VIM-2 variants are the most prevalent, commonly found in clinically relevant Gram-negatives as non-fermenters *Pseudomonas* spp. and *Acinetobacter* spp. and *Enterobacteriaceae* members (Nordmann and Poirel, 2014; Patel and Bonomo, 2013; Queenan and Bush, 2007). In Portugal, other variants of IMP (mostly IMP-5) have been detected both in nosocomial and environmental strains (Da Silva et al.,

2002; Kieffer et al., 2016). VIM-2 has been reported in *P. aeruginosa* (Botelho et al., 2018; Cardoso et al., 2002; Quinteira et al., 2005; Quinteira and Peixe, 2006), *Klebsiella oxytoca* (Conceição et al., 2005) and recently in *C. freundii* (Santos et al., 2017). Since the discovery of NDM-1 (Yong et al., 2009), NDM mobilizable enzymes accelerated spread has been reported all over the world and more worrisome is their ubiquity and association with other resistance genes (R. Cantón et al., 2012; Kilic and Baysallar, 2015; Poirel et al., 2010b, 2011; Zhang et al., 2013) including *mcr-1* (Du et al., 2016). In Portugal only two detections have occurred until now; in an opportunistic *Providencia stuartii* producing NDM-1 isolate (Manageiro et al., 2015b) and in an environmental *Enterobacter roggenkampii* isolated from a river (Teixeira et al., 2020).

Similar to the other β-lactamases, MBLs were initially constrained geographically and the first ones detected were chromosomal (Kuwabara and Abraham, 1969; Saino et al., 1982). In contrast to the chromosomally-encoded MBLs, there has been a dramatic increase in the detection and spread of the acquired and transferable families of these metallo-enzymes, by the continued use of carbapenems as the number of infections caused by ESBL-producers increased (Guh et al., 2015; Kelly et al., 2017; Patel and Bonomo, 2013), both in clinical settings (Nordmann et al., 2011) and livestock/environment, even if sporadically (Köck et al., 2018). This class of enzymes is characterized by a quite broad spectrum with ability to hydrolyze carbapenems, cephalosporins, and penicillins and β-lactamase inhibitors, but are inhibited by monobactams, and metal chelators (Queenan and Bush, 2007). Although these enzymes lack activity against monobactams such as aztreonam, they can confer high level of resistance when combined with alterations in membrane permeability and ESBL co-production (Bush, 2010; Queenan and Bush, 2007).

Serine β-lactamases from molecular class A include the most clinically relevant and widely disseminated KPC enzymes (*K. pneumoniae* carbapenemases) but also less frequent enzymes such as SFC-1 and SHV-38, SME, NMC-A/IMI and a subgroup of the GES family (Henriques et al., 2004; Nordmann and Poirel, 2014; Poirel et al., 2003a). Chromosomally-encoded class A serine carbapenemases are to these days still rarely isolated and geographically restrained, while plasmid-encoded variants of KPC and GES family enzymes are disseminated throughout the globe with more than 40 known variants (Bush, 2018b; NCBI, 2016; Queenan

and Bush, 2007). Bacteria expressing class A serine carbapenemases have the ability to hydrolyze a broad variety of β-lactams, including carbapenems (susceptibility to imipenem while reduced it is measurable), cephalosporins, penicillins, and aztreonam, and are all suppressed by β-lactamase inhibitors clavulanic acid and tazobactam (Queenan and Bush, 2007).

Class D serine carbapenemases, also known as OXA enzymes, as the name says cleave oxacillin in addition to penicillin, thus distinguishing them from class A β-lactamases. This group of enzymes consists of a very diverse family of chromosomally- and plasmid-encoded enzymes due to a large amount of variability in their amino acid sequences. Mostly identified among *Acinetobacter* spp. (Mathlouthi et al., 2015; Patel and Bonomo, 2013), but increasingly isolated in *Enterobacteriaceae* (Patel and Bonomo, 2013; Poirel et al., 2012; Potron et al., 2011a; A. Potron et al., 2013; Tacão et al., 2018), $bla_{\text{OXA-48}}$-like acquired genes have also been detected in *P. aeruginosa* (Mathlouthi et al., 2015; Meunier et al., 2016). OXA-48 variants have been reported particularly in *Enterobacteriaceae* not only in clinical contexts but also in environmental compartments (Galler et al., 2014; Potron et al., 2011b). Molecular classes A and D, represent the largest group of β-lactamases, due primarily to the increasing identification of ESBLs during the past decades (Bush and Jacoby, 2010) and OXA enzymes increasing global frequency in the last decade (Potter et al., 2016).

3. AR IN THE ENVIRONMENT AND THE ANTHROPOGENIC EFFECT

In the past decades, bacterial resistance to antimicrobial compounds has developed almost entirely due to men's action (Surette and Wright, 2017). An extreme scenario of this anthropogenic effect are antibiotic production facilities, which release substantial amounts of drugs into the environment reaching levels 1,000 times higher than concentrations typically used to kill bacteria in healthcare settings (Larsson et al., 2007; Pal et al., 2016). Another example can be the microbiomes of built environments like hospital settings where there is a strong correlation with AR on all kinds of surfaces and even air (Leung and Lee, 2016).

Concomitantly, as environmental organisms produce secondary metabolites such as (e.g.) antibiotics and must also evolve mechanisms to protect themselves from the toxic activity of these molecules (Cundliffe, 1992), the *natural* environment plays as a hotspot for the evolution of AR. Similarly, due to the release of antibiotics in the environment by naturally occurring soil-dwelling bacteria, their microbial neighbors – not innately antibiotic producers – must co-evolve coping strategies to compete for resources, and to survive, ultimately. This can occur either through the evolution of resistance mechanisms in the microbial neighbor or by HGT of resistance genes from other bacterial species (Surette and Wright, 2017).

For that matter, environmental habitats are reservoirs for resistance. Bacteria from all kinds of different ecosystems are potential suppliers for the resistome of the ecological compartment in which they are included in, being it from the *natural* environment or either from impacted-by-man or man-made environment (Figure 2).

Soils are highly variable ecosystems, dependent on water content, mineral composition, oxygen concentration, and nutrient availability and with a diversity of living organisms (Surette and Wright, 2017). Soil microorganisms live closely with each other on a micron scale. Hence, producers of bioactive compounds, such as *Streptomyces* and other actinomycetes and filamentous fungi, have their

microbial neighbors harboring genetically diverse resistomes evolved to attenuate antibiotic killing activity (Katz and Blatz, 2016, Surette and Wright, 2017). Moreover, even though there is some doubts about these secreted metabolites' primary role, which are believed to be involved in multiple effects on adjacent cell metabolism and gene expression (Surette and Bernier, 2013); very likely, many do have antimicrobial activity. In fact, as proximity of cells increases a gradient of antibiotic concentration is observed, higher close to the producing cells that diminishes as compounds diffuse (Traxler, 2013).

Airborne bacteria can travel across continents in particles of dust or aerosols (Creamean et al., 2013). The resistance burden in atmospheric microbial communities, although low, is detectable (Mazar et al., 2016). Contrarily to unpolluted air, in highly urbanized areas smog and antibiotic-polluted environments such as farms and highly dense urban areas, diverse AR elements are readily identified in air samples (Gao et al., 2016; Pal et al., 2016).

Like air and soils, aquatic environments are also very well recognized reservoirs of antibiotic-resistant bacteria (ARB) and ARGs (Kümmerer, 2009a, 2009b; Marti et al., 2014; O'Flaherty and Cummins, 2017). Although the lower bacterial densities in comparison to soils, their sediments are, likewise, rich in bacterial diversity. Several studies have pointed out the increased resistance burden when in proximity to human activities, in particular, pharmaceutical and other polluting industries, in which resistance elements and MDR bacteria are found at levels much higher than those observed in pristine locations (Pal et al., 2016; Tacão et al., 2012). Additionally, urban wastewater treatment plants (UWTPs) are important interfaces between the human population and the environment (O'flaherty et al., 2018) as they are considered point sources of accumulated and diverse emerging pollutants, including antibiotics, ARB and ARGs (Rizzo et al., 2013). UWTPs are for this reason very crucial receptors and sources of environmental AR, often seen as hotspots for AR dissemination due to the resistance burden observed in these environments (Pärnänen et al., 2019; Wu et al., 2019). Also, HGT between genetically distinct bacteria can be promoted depending on cell movement in water films (Harshey, 2003).

Finally, while agriculture activities are beyond the environmental scope they are intimately linked to the environmental drug resistance burden. Facilities of

animal husbandry often employ astonishing amounts of antimicrobials for growth promotion and infection control and are extremely fitting environments for the accumulation and spread of mobile genetic elements. Globally, according to the WHO most antibiotics are used in livestock resulting in a massive expansion and mobilization of AR in the bacteria that reside on and in these animals (WHO – Regional Office for Europe, 2011). By 2014, about 70% of all antibiotics sold in the EU were for use in food-producing animals (ECDC/EFSA/EMA, 2017). Moreover, agricultural practices such as the application of manure and sewage sludge in fields can significantly alter the composition of soil resistomes (Chen et al., 2016; Graham et al., 2016; Marti et al., 2013). Such practices promote the enrichment of soils with genes often found in pathogens, thereby substantially mixing human and environmental resistomes and, thus, promoting that ARB from animals microbiome serve as a reservoir of clinically important ARGs (de Been et al., 2014).

4. DISSEMINATION OF AR – CLINIC VS. ENVIRONMENT

AR dissemination is the main reason bacterial infectious diseases are becoming more severe and require longer and more expensive treatments. In fact, infectious diseases that were formerly considered to be eradicated in developed countries, such as tuberculosis and gonorrhea, are now present and a real threat (Laxminarayan, 2014). Thus, the dissemination of pathogenic strains resistant to multiple antibiotics presents an enormous concern, challenging clinical practices.

Most researchers from the field considerably believe that AR in clinical settings sprouted from environmental bacteria (D'Costa, 2006; Davies and Davies, 2010; Forsberg et al., 2012; Poirel et al., 2005b; Surette and Wright, 2017); Albeit in some cases is not necessarily linked to exposure and misuse of antibiotics (Cox and Wright, 2013) and the inverse process also happens (Fajardo et al., 2008; Jasper et al., 2015). Their application, however, is assented by all as the fuel that enhanced and perpetuates the problem, providing selective pressure for the random capture of ARGs by mobile genetic elements that eventually can be captured by pathogens (Surette and Wright, 2017). There are also other lines of

evidence suggesting that many ARGs found in pathogens today, have an environmental origin (Poirel et al., 2005; Tacão et al., 2018), clearly emphasizing the importance of environmental bacteria as potential sources for clinically important forms of resistance.

AR in the environment is ancient and predates the use of antibiotics. Recent studies provided evidence that most (perhaps all) environmental bacterial genomes harbor AR elements, many in the form of intrinsic mechanisms (Cox and Wright, 2013; D'Costa, 2006). Furthermore, many bacteria display a variety of genes and pseudogenes encoding integrases and transposases; marks confirming a long history of gene mobilization that remain to offer facile routes of HGT within and across microbial species and genera (Soucy et al., 2015). As mentioned before, while human activity correlates with high levels of AR, ARGs ancestors and genes similar to known contemporary ARGs can be found in remote environments with minimal anthropogenic impact across the globe, such as 30,000-year-old permafrost (D'Costa et al., 2011), isolated caves (Bhullar et al., 2012), Alaskan soil (Allen et al., 2009) and glaciers (Segawa et al., 2013) and Red Sea brine pools (Elbehery et al., 2017).

The spread of AR is of global health concern. A 'One Health' perspective is urgent as AR emergence and dissemination involves a dynamic and complex web of interactions, where there are many paths by which ARB and ARG can disseminate between humans, animals, and the environment (Figure 2) (Harbarth et al., 2015). While the highest concentrations of antibiotics have been recorded in effluents released from hospitals and drug manufacturing sites in developing countries (Larsson et al., 2007; Pal et al., 2016), wastewater treatment plants are also another large contributor for releasing drug residues, resistant bacteria, and genes into the environment, as many studies have demonstrated (Manaia et al., 2016; Marathe et al., 2019; Michael et al., 2013; Pärnänen et al., 2019; Rizzo et al., 2013; Silva et al., 2018).

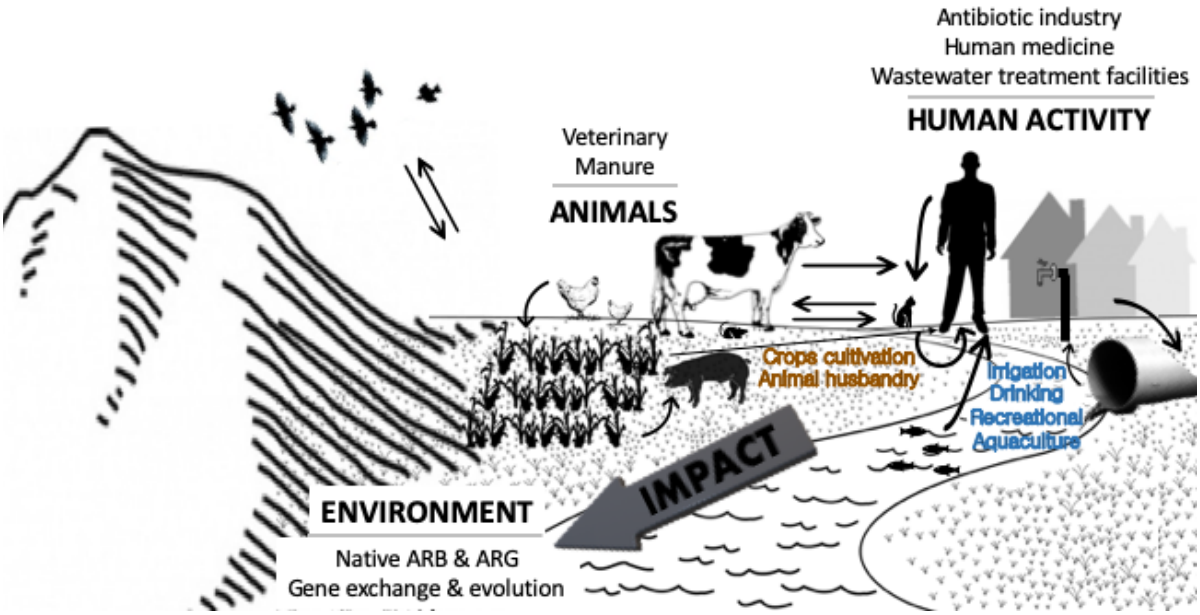


FIGURE 2. Sources and movement of antibiotic resistant bacteria and antibiotic resistance genes in the environment and the impact of anthropogenic activities.

Other routes of dissemination of AR can be through the food chain or contact with animals. For example, resistance in zoonotic foodborne species is clearly linked to antibiotics use in food animals, and foodborne diseases caused by such resistant bacteria are well documented in humans, as the case of *Salmonella* and *Campylobacter* bacteria that developed resistance to a fluoroquinolone similar to one commonly used in humans (WHO – Regional Committee for Europe, 2011). Similarly, occurs in foodborne *E. coli* as it is frequently found in retail meat (Collignon, 2009; Johnson et al., 2009; Szmolka and Nagy, 2013) and often associated to vegetable consumption outbreaks (Friesema et al., 2008; Söderström et al., 2008). In certain cases, drinking water can also play a role in dissemination, as demonstrated by carbapenem-resistant bacteria detected in chlorinated water samples from New Delhi distribution system (Tanner et al., 2015) or untreated drinking water from pristine environments (Henriques et al., 2004, 2012; Saavedra et al., 2003).

Animal-associated infections in humans are not so common, yet the fact that the antibiotics used in human and animal health largely comprise the same or very similar molecules would be expected to drive the transmission of resistance between animals and people, either directly or via the environment. Moreover, the way that most antibiotics are used in animal production in sub-therapeutic

doses and for long exposure periods creates ideal conditions for bacteria to fix genes that confer resistance. Important to note is that, even if some bacteria are species (animal)-specific and present a low risk of infection to humans, the ARGs potentially carried by these strains are likely to be much less host-specific and hence transferrable to bacteria carried more frequently by humans (Collignon, 2009). In the case of CRE, transmission between animals and humans in either direction has been pointed as a risk for public health, as the occurrence of foodborne or zoonotic CRE cases has been detected in clinical context with origin in food-producing animals, pets, wildlife, especially in regions considered endemic of certain CRE, like India and China on Asia, and Northern Africa (Köck et al., 2018; Liu et al., 2016).

The more recent alarming case of resistance associated with antimicrobial usage on food animal production, with high risk to human health, is plasmid-encoded colistin resistance mediated by the MCR-1 protein, that was unexpectedly identified in *E. coli* isolates from a pig during a routine surveillance program in China (Liu et al., 2016). Soon after being identified by the pioneer Chinese work, the *mcr-1* gene was identified in isolates from animals, humans, food, and environmental samples all over the world (Nordmann and Poirel, 2016). The *mcr-1*-containing plasmid has capabilities to transfer into epidemic strains, such as *E. coli* ST131 and *K. pneumoniae* ST11, as well as into *P. aeruginosa*, hence suggesting it is likely to spread rapidly into key human pathogens due to its interspecies transferability (Liu et al., 2016; Nordmann and Poirel, 2016). An important note is the fact that until the acquired resistance finding, polymyxins use was restricted to animal-farming. An aggravation of the resistance to this last resort antibiotic mediated by *mcr-1*-harboring plasmids is the co-occurrence of other ARGs in the same isolate, as bla_{KPC-3} was reported in the pEc36-KPC3 plasmid in Portugal, also co-harboring bla_{TEM}, bla_{OXA-9}, *aacA4*, and *aadA1* ARGs (Tacão et al., 2017), and other important carbapenemases such as VIM (Poirel et al., 2016), NDM (Du et al., 2016) and OXA-48 (Pulss et al., 2017), which demonstrates the alarming scenario around this relatively novel and highly mobilizable gene.

Resistance to antibiotics is not surprising, but the current state of resistance against vital antibiotics and its acquisition by commensal bacteria is quite worrisome. Elements that enable bacteria to neutralize the toxic effects of an otherwise effective drug are attributed to the genetic elements that are either chromosomally-encoded and thus vertically transmitted or acquired horizontally from the environment. The latter case asks for more attention, as it is usually associated with transferable elements and co-resistance to other important drugs, which means that resistance can arise due to a multiplicity of selective pressures. In the past, it was already confirmed by strong evidence that clinical resistance genes have direct links to the environmental resistome. By instance, aminoglycoside-modifying enzymes were proven to be originated from soil-dwelling actinomycetes. Species of *Streptomyces* genus were identified encoding two molecular mechanisms, inactivation by acetylation and phosphorylation kanamycin and neomycin, respectively, as a means of self-protection as they are producers themselves (Benveniste and Davies, 1973). In the case of vancomycin resistance, the *vanHAX* genes cluster encoding for VanH-VanA-VanX proteins was discovered also among soil actinomycetes. Vancomycin resistance emerged into the clinic through *Enterococcus* bacteria (vancomycin-resistant enterococci – VRE) and then to *Staphylococcus aureus* (vancomycin-resistant *S. aureus* – VRSA) (Courvalin, 2006; D'Costa, 2006; D'Costa et al., 2007; Wright, 2007). The evolution of the transfer of the genes that encode for these resistance mechanisms, from the environmental progenitors to clinical relevant bacteria, have yet a defined conclusion. There are several plausible hypothesis, that with increasing numbers of genome sequencing in recent years can shed light on the knowledge gap. Other example of clinical resistance genes with environmental origins are the widely disseminated plasmid-mediated CTX-Ms of significant clinical impact discovered in chromosome-encoded genes of *Kluyvera* spp. which, along multiple mobilization elements but especially IS*Ecp1* and IS*CR1* determinants in the plasmid-harbored $bla_{\text{CTX-M}}$ gene surrounding, were transferred to other bacteria (Rafael Cantón et al., 2012; Poirel et al., 2002; Zhao and Hu, 2013). Intrinsic carbapenemase OXA-48 producer was putatively found to be waterborne *Shewanella* spp. (Poirel et al., 2012, 2004; Potron et al., 2011a) which was mobilized through ISs into plasmids and possibly had an intermediate reservoir (Potron et al., 2011b). Another example is the quinolone resistance gene *qnrB* in *Citrobacter* spp.. Ribeiro and co-workers suggest a potential route of

mobilization of these genes into the clinical settings, in which the plasmid-associated genetic surroundings are thought to occur by multiple arrangements and transfer mechanisms (Ribeiro et al., 2015). In the absence of new antimicrobial agents effective against resistant Gram-negative pathogens, the effect on human health cannot be underestimated.

It is imperative that surveillance and molecular epidemiological studies on the distribution and dissemination of such resistance genes among Gram-negative bacteria in both human and veterinary medicine are initiated, along with re-evaluation of the use of antibiotics across all applicable settings (Magiorakos et al., 2017; Topp et al., 2017).

The findings herein exposed thus raise warnings, underlining the importance of implementing measures in order to mitigate the spread of AR in the environment, particularly under the 'One Health' perspective.

5. REFERENCES

Abbas, M., Paul, M., Huttner, A., 2017. New and improved? A review of novel antibiotics for Gram-positive bacteria. Clin. Microbiol. Infect. 23, 697–703. doi:10.1016/j.cmi.2017.06.010

Abraham, E.P., Chain, E., 1940. An enzyme from bacteria able to destroy penicillin. Rev. Infect. Dis. 10, 677–8.

Adegoke, A.A., Stenström, T.A., Okoh, A.I., 2017. *Stenotrophomonas maltophilia* as an emerging ubiquitous pathogen: Looking beyond contemporary antibiotic therapy. Front. Microbiol. 8, 2276. doi:10.3389/fmicb.2017.02276

Allen, H.K., Moe, L.A., Rodbumrer, J., Gaarder, A., Handelsman, J., 2009. Functional metagenomics reveals diverse β-lactamases in a remote Alaskan soil. ISME J. 3, 243–251. doi:10.1038/ismej.2008.86

Alvarez, M., Tran, J.H., Chow, N., Jacoby, G.A., 2004. Epidemiology of conjugative plasmid-mediated AmpC β-Lactamases in the United States. Antimicrob. Agents Chemother. 48, 533–537. doi:10.1128/AAC.48.2.533-537.2004

Alves, M.S., Pereira, A., Araújo, S.M., Castro, B.B., Correia, A.C.M, Henriques, I., 2014. Seawater is a reservoir of multi-resistant *Escherichia coli*, including strains hosting plasmid-mediated quinolones resistance and extended-spectrum beta-lactamases genes. Front. Microbiol. 5, 1–10. doi:10.3389/fmicb.2014.00426

Ambler, R.P., 1980. The structure of β-lactamases. Philos. Trans. R. Soc. B Biol. Sci. 289, 321–331. doi:10.1098/rstb.1980.0049

Avison, M.B., Higgins, C.S., von Heldreich, C.J., Bennett, P.M., Walsh, T.R., 2001. Plasmid location and molecular heterogeneity of the L1 and L2 β-lactamase genes of *Stenotrophomonas maltophilia*. Antimicrob. Agents Chemother. 45, 413–419. doi:10.1128/AAC.45.2.413

Bassetti, M., Peghin, M., Vena, A., Giacobbe, D.R., 2019. Treatment of infections due to MDR Gram-negative bacteria. Front. Med. 6, 1–10. doi:10.3389/fmed.2019.00074

Bauernfeind, A., Stemplinger, I., Jungwirth, R., Mangold, P., Amann, S., Akalin, E., Anğ, Ö., Bal, C., Casellas, J.M., 1996. Characterization of β-lactamase gene

bla$_{PER-2}$, which encodes an extended-spectrum class A β-lactamase. Antimicrob. Agents Chemother. 40, 616–620. doi:10.1128/aac.40.3.616

Beceiro, A., Maharjan, S., Gaulton, T., Doumith, M., Soares, N.C., Dhanji, H., Warner, M., Doyle, M., Hickey, M., Downie, G., Bou, G., Livermore, D.M., Woodford, N., 2011. False extended-spectrum β-lactamase phenotype in clinical isolates of *Escherichia coli* associated with increased expression of OXA-1 or TEM-1 penicillinases and loss of porins. J. Antimicrob. Chemother. 66, 2006–2010. doi:10.1093/jac/dkr265

Benveniste, R., Davies, J., 1973. Aminoglycoside antibiotic inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic resistant bacteria. Proc. Natl. Acad. Sci. U.S.A. 70, 2276–2280. doi:10.1073/pnas.70.8.2276

Bernier, S.P., Surette, M.G., 2013. Concentration-dependent activity of antibiotics in natural environments. Front. Microbiol. 4. doi:10.3389/fmicb.2013.00020

Bhullar, K., Waglechner, N., Pawlowski, A., Koteva, K., Banks, E.D., Johnston, M.D., Barton, H.A., Wright, G.D., 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS One 7, e34953. doi:10.1371/journal.pone.0034953

Botelho, J., Grosso, F., Quinteira, S., Brilhante, M., Ramos, H., Peixe, L., 2018. Two decades of *bla*$_{VIM-2}$-producing *Pseudomonas aeruginosa* dissemination: An interplay between mobile genetic elements and successful clones. J. Antimicrob. Chemother. 73, 873–882. doi:10.1093/jac/dkx517

Bradford, P.A., 2001. Extended-spectrum β-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. doi:10.1128/CMR.14.4.933-951.2001

Bush, K., 2018a. Game Changers: New β-Lactamase Inhibitor Combinations Targeting Antibiotic Resistance in Gram-Negative Bacteria. ACS Infect. Dis. 4, 84–87. doi:10.1021/acsinfecdis.7b00243

Bush, K., 2018b. Past and Present Perspectives on β-Lactamases. Antimicrob. Agents Chemother. 62. doi:10.1128/aac.01076-18

Bush, K., 2010. Alarming β-lactamase-mediated resistance in multidrug-resistant *Enterobacteriaceae*. Curr. Opin. Microbiol. doi:10.1016/j.mib.2010.09.006

Bush, K., Bradford, P.A., 2019. Interplay between β-lactamases and new β-

lactamase inhibitors. Nat. Rev. Microbiol. doi:10.1038/s41579-019-0159-8

Bush, K., Bradford, P.A., 2016. β-lactams and β-lactamase inhibitors: An overview. Cold Spring Harb. Perspect. Med. 6, a025247. doi:10.1101/cshperspect.a025247

Bush, K., Jacoby, G.A., 2010. Updated functional classification of β-lactamases. Antimicrob. Agents Chemother. 54, 969–976. doi:10.1128/AAC.01009-09

Bush, K., Jacoby, G.A., Medeiros, A.A., 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. 39, 1211–1233. doi:10.1128/AAC.39.6.1211

Cantón, R., Akóva, M., Carmeli, Y., Giske, C.G., Glupczynski, Y., Gniadkowski, M., Livermore, D.M., Miriagou, V., Naas, T., Rossolini, G.M., Samuelsen, Seifert, H., Woodford, N., Nordmann, P., Poirel, L., Bogaerts, P., Navon-Venezia, S., Cornaglia, G., 2012. Rapid evolution and spread of carbapenemases among *Enterobacteriaceae* in Europe. Clin. Microbiol. Infect. 18, 413–431. doi:10.1111/j.1469-0691.2012.03821.x

Cantón, Rafael, González-Alba, J.M., Galán, J.C., 2012. CTX-M enzymes: Origin and diffusion. Front. Microbiol. doi:10.3389/fmicb.2012.00110

Carattoli, A., 2013. Plasmids and the spread of resistance. Int. J. Med. Microbiol. doi:10.1016/j.ijmm.2013.02.001

Carattoli, A., 2009. Resistance plasmid families in *Enterobacteriaceae*. Antimicrob. Agents Chemother. 53, 2227–2238. doi:10.1128/AAC.01707-08

Cardoso, O., Leitão, R., Figueiredo, A., Sousa, J.C., Duarte, A., Peixe, L.V., 2002. Metallo-β-lactamase VIM-2 in clinical isolates of *Pseudomonas aeruginosa* from Portugal. Microb. Drug Resist. 8, 93–97. doi:10.1089/107662902760190635

Cassini, A., Högberg, L.D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G.S., Colomb-Cotinat, M., Kretzschmar, M.E., Devleesschauwer, B., Cecchini, M., et al, 2019. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect. Dis. 19, 56–66. doi:10.1016/S1473-3099(18)30605-4

Centers for Disease Control and Prevention (CDC), 2013. Antibiotic resistance threats in the United States, 2013. doi:CS239559-B

Centers for Disease Control and Prevention (CDC), 2011. Ten Great Public Health Achievements - Worldwide, 2001–2010.

Chambers, H.F., 1999. Penicillin-binding protein-mediated resistance in pneumococci and staphylococci. J. Infect. Dis. doi:10.1086/513854

Chen, Q., An, X., Li, H., Su, J., Ma, Y., Zhu, Y.G., 2016. Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil. Environ. Int. doi:10.1016/j.envint.2016.03.026

Collignon, P., 2009. Resistant *Escherichia coli* – We are what we eat. Clin. Infect. Dis. 49, 202–204. doi:10.1086/599831

Conceição, T., Brízio, A., Duarte, A., Barros, R., 2005a. First isolation of bla_{VIM-2} in *Klebsiella oxytoca* clinical isolates from Portugal. Antimicrob. Agents Chemother. doi:10.1128/AAC.49.1.476.2005

Conceição, T., Brízio, A., Duarte, A., Lito, L.M., Cristino, J.. M., Salgado, M.. J., 2005b. First description of CTX-M-15-producing *Klebsiella pneumoniae* in Portugal. Antimicrob. Agents Chemother. 49, 477–478. doi:10.1128/AAC.49.1.477-478.2005

Costa, D., Poeta, P., Sáenz, Y., Vinué, L., Rojo-Bezares, B., Jouini, A., Zarazaga, M., Rodrigues, J., Torres, C., 2006. Detection of *Escherichia coli* harbouring extended-spectrum β-lactamases of the CTX-M, TEM and SHV classes in faecal samples of wild animals in Portugal. J. Antimicrob. Chemother. doi:10.1093/jac/dkl415

Courvalin, P., 2006. Vancomycin resistance in Gram-positive cocci. Clin. Infect. Dis. doi:10.1086/491711

Cox, G., Wright, G.D., 2013. Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions. Int. J. Med. Microbiol. 303, 287–292. doi:10.1016/j.ijmm.2013.02.009

Creamean, J.M., Suski, K.J., Rosenfeld, D., Cazorla, A., DeMott, P.J., Sullivan, R.C., White, A.B., Ralph, F.M., Minnis, P., Comstock, J.M., Tomlinson, J.M., Prather, K.A., 2013. Dust and biological aerosols from the Sahara and Asia influence precipitation in the Western U.S. Science. 339, 1572–1578. doi:10.1126/science.1227279

Cundliffe, E., 1992. Self-protection mechanisms in antibiotic producers. Ciba Found. Symp. doi:10.1002/9780470514344.ch12

D'Andrea, M.M., Arena, F., Pallecchi, L., Rossolini, G.M., 2013. CTX-M-type β-lactamases: A successful story of antibiotic resistance. Int. J. Med. Microbiol. doi:10.1016/j.ijmm.2013.02.008

D'Costa, V.M., 2006. Sampling the antibiotic resistome. Science (80-.). 311, 374–377. doi:10.1126/science.1120800

D'Costa, V.M., Griffiths, E., Wright, G.D., 2007. Expanding the soil antibiotic resistome: exploring environmental diversity. Curr. Opin. Microbiol. 10, 481–489. doi:10.1016/J.MIB.2007.08.009

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., Wright, G.D., 2011. Antibiotic resistance is ancient. Nature 477, 457–461. doi:10.1038/nature10388

Da Silva, G.J., Correia, M., Vital, C., Ribeiro, G., Sousa, J.C., Leitão, R., Peixe, L., Duarte, A., 2002. Molecular characterization of bla_{IMP-5}, a new integron-borne metallo-β-lactamase gene from an *Acinetobacter baumannii* nosocomial isolate in Portugal. FEMS Microbiol. Lett. 215, 33–39. doi:10.1016/S0378-1097(02)00896-0

Davies, J., 1995. Vicious circles: Looking back on resistance plasmids Julian. Genetics. 139, 1465–1468.

Davies, J., Davies, D., 2010. Origins and evolution of antibiotic resistance. Microbiol. Mol. Biol. 74, 417–433. doi:10.1128/MMBR.00016-10

de Been, M., Lanza, V.F., de Toro, M., Scharringa, J., Dohmen, W., Du, Y., Hu, J., Lei, Y., Li, N., Tooming-Klunderud, A., Heederik, D.J.J., Fluit, A.C., Bonten, M.J.M., Willems, R.J.L., de la Cruz, F., van Schaik, W., 2014. Dissemination of cephalosporin resistance genes between *Escherichia coli* strains from farm animals and humans by specific plasmid lineages. PLoS Genet. 10, 1004776. doi:10.1371/journal.pgen.1004776

Drawz, S.M., Bonomo, R.A., 2010. Three decades of β-lactamase inhibitors. Clin. Microbiol. Rev. 23, 160–201. doi:10.1128/CMR.00037-09

Du, H., Chen, L., Tang, Y.W., Kreiswirth, B.N., 2016. Emergence of the *mcr-1* colistin resistance gene in carbapenem-resistant *Enterobacteriaceae*. Lancet Infect. Dis. doi:10.1016/S1473-3099(16)00056-6

European Center for Disease Prevention and Control, European Food Safety

Authority, European Medicines Agency, 2017. ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals, EFSA Journal. doi:10.2903/j.efsa.2017.4872

Elbehery, A.H.A., Leak, D.J., Siam, R., 2017. Novel thermostable antibiotic resistance enzymes from the Atlantis II Deep Red Sea brine pool. Microb. Biotechnol. 10, 189–202. doi:10.1111/1751-7915.12468

European Center for Disease Prevention and Control, European Food Safety Authority, European Medicines Agency, 2015. ECDC/EFSA/EMA first joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals, EFSA Journal. doi:10.2903/j.efsa.2015.4006

European Parliament and the Council, 2018. Regulation (EU) 2019/ of the European Parliament and of the Council of 11 December 2018 on veterinary medicinal products and repealing Directive 2001/82/EC. Off. J. Eur. Union.

Evans, B.A., Amyes, S.G.B., 2014. OXA β-Lactamases. Clin. Microbiol. Rev. 27, 241–263. doi:10.1128/CMR.00117-13

Fajardo, A., Martínez-Martín, N., Mercadillo, M., Galán, J.C., Ghysels, B., Matthijs, S., Cornelis, P., Wiehlmann, L., Tümmler, B., Baquero, F., Martínez, J.L., 2008. The neglected intrinsic resistome of bacterial pathogens. PLoS One 3. doi:10.1371/journal.pone.0001619

Falagas, M.E., Rafailidis, P.I., Matthaiou, D.K., 2010. Resistance to polymyxins: Mechanisms, frequency and treatment options. Drug Resist. Updat. 13, 132–138. doi:10.1016/j.drup.2010.05.002

Faour-Klingbeil, D., Murtada, M., Kuri, V., Todd, E.C.D., 2016. Understanding the routes of contamination of ready-to-eat vegetables in the Middle East. Food Control 62, 125–133. doi:10.1016/j.foodcont.2015.10.024

Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A., Dantas, G., 2012. The shared antibiotic resistome of soil bacteria and human pathogens. Science (80-.). 337, 1107–1111. doi:10.1126/science.1220761

Friesema, I., Sigmundsdottir, G., van der Zwaluw, K., Heuvelink, A., Schimmer, B., de Jager, C., Rump, B., Briem, H., Hardardottir, H., Atladottir, A., Gudmundsdottir, E., van Pelt, W., 2008. An international outbreak of Shiga toxin-producing *Escherichia coli* O157 infection due to lettuce, September -

October 2007. Eurosurveillance 13, 1–5.

Galler, H., Feierl, G., Petternel, C., Reinthaler, F.F., Haas, D., Grisold, A.J., Luxner, J., Zarfel, G., 2014. KPC-2 and OXA-48 carbapenemase-harbouring *Enterobacteriaceae* detected in an Austrian wastewater treatment plant. Clin. Microbiol. Infect. 20, O132–O134. doi:10.1111/1469-0691.12336

Gao, X.L., Shao, M.F., Luo, Y., Dong, Y.F., Ouyang, F., Dong, W.Y., Li, J., 2016. Airborne bacterial contaminations in typical Chinese wet market with live poultry trade. Sci. Total Environ. 572, 681–687. doi:10.1016/j.scitotenv.2016.06.208

Garcês, A., Correia, S., Amorim, F., Pereira, J.E., Igrejas, G., Poeta, P., 2019. First report on extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* from European free-tailed bats (*Tadarida teniotis*) in Portugal: A one-health approach of a hidden contamination problem. J. Hazard. Mater. 370, 219–224. doi:10.1016/j.jhazmat.2017.12.053

Girlich, D., Naas, T., Leelaporn, A., Poirel, L., Fennewald, M., Nordmann, P., 2002. Nosocomial spread of the integron-located veb-1-like cassette encoding an extended-spectrum β-lactamase in *Pseudomonas aeruginosa* in Thailand. Clin. Infect. Dis. 34, 603–611. doi:10.1086/338786

Graham, D.W., Knapp, C.W., Christensen, B.T., McCluskey, S., Dolfing, J., 2016. Appearance of β-lactam resistance genes in agricultural soils and clinical isolates over the 20th century. Sci. Rep. 6. doi:10.1038/srep21550

Guh, A.Y., Bulens, S.N., Mu, Y., Jacob, J.T., Reno, J., Scott, J., Wilson, L.E., Vaeth, E., Lynfield, R., Shaw, K.M., Vagnone, P.M.S., Bamberg, W.M., Janelle, S.J., Dumyati, G., Concannon, C., Beldavs, Z., Cunningham, M., Cassidy, P.M., Phipps, E.C., Kenslow, N., Travis, T., Lonsway, D., Rasheed, J.K., Limbago, B.M., Kallen, A.J., 2015. Epidemiology of carbapenem-resistant *Enterobacteriaceae* in 7 US communities, 2012-2013. J. Am. Med. Assoc. 314, 1479–1487. doi:10.1001/jama.2015.12480

Harbarth, S., Balkhy, H.H., Goossens, H., Jarlier, V., Kluytmans, J., Laxminarayan, R., Saam, M., Van Belkum, A., Pittet, D., 2015. Antimicrobial resistance: One world, one fight! Antimicrob. Resist. Infect. Control 4, 49. doi:10.1186/s13756-015-0091-2

Harshey, R.M., 2003. Bacterial motility on a surface: Many ways to a common goal. Annu. Rev. Microbiol. 57, 249–273. doi:10.1146/annurev.micro.57.030502.091014

Henriques, I., Moura, A., Alves, A., Saavedra, M.J., Correia, A., 2004. Molecular characterization of a carbapenem-hydrolyzing class A β-lactamase, SFC-1, from *Serratia fonticola* UTAD54. Antimicrob. Agents Chemother. 48, 2321–2324. doi:10.1128/AAC.48.6.2321-2324.2004

Henriques, I.S.I.S., Araújo, S., Azevedo, J.S.N.J.S.N., Alves, M.S.M.S., Chouchani, C., Pereira, A., Correia, A., 2012. Prevalence and diversity of carbapenem-resistant bacteria in untreated drinking water in Portugal. Microb. Drug Resist. 18, 531–537. doi:10.1089/mdr.2012.0029

Hoelzer, K., Wong, N., Thomas, J., Talkington, K., Jungman, E., Coukell, A., 2017. Antimicrobial drug use in food-producing animals and associated human health risks: what, and how strong, is the evidence? BMC Vet. Res. 13, 211. doi:10.1186/s12917-017-1131-3

Humeniuk, C., Arlet, G., Gautier, V., Grimont, P., Labia, R., Philippon, A., 2002. β-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. Antimicrob. Agents Chemother. doi:10.1128/AAC.46.9.3045-3049.2002

Jacoby, G.A., 2009. AmpC β-lactamases. Clin. Microbiol. Rev. 22, 161–182. doi:10.1128/CMR.00036-08

Jasper, R.T., Coyle, J.R., Katz, D.E., Marchaim, D., 2015. The complex epidemiology of extended-spectrum β-lactamase-producing *Enterobacteriaceae*. Future Microbiol. 10, 819–839.

Johnson, J.R., McCabe, J.S., White, D.G., Johnston, B., Kuskowski, M.A., McDermott, P., 2009. Molecular analysis of *Escherichia coli* from retail meats (2002–2004) from the United States National Antimicrobial Resistance Monitoring System. Clin. Infect. Dis. 49, 195–201. doi:10.1086/599830

Katz, L., Baltz, R.H., 2016. Natural product discovery: past, present, and future. J. Ind. Microbiol. Biotechnol. 43, 155–176. doi:10.1007/s10295-015-1723-5

Kelly, A.M., Mathema, B., Larson, E.L., 2017. Carbapenem-resistant *Enterobacteriacea*e in the community: a scoping review. Int. J. Antimicrob. Agents 50, 127–134. doi:10.1016/j.ijantimicag.2017.03.012

Kieffer, N., Poirel, L., Bessa, L.J., Barbosa-Vasconcelos, A., Da Costa, P.M., Nordmann, P., 2016. VIM-1, VIM-34, and IMP-8 carbapenemase-producing *Escherichia coli* strains recovered from a Portuguese river. Antimicrob. Agents Chemother. 60, 2585–2586. doi:10.1128/AAC.02632-15

Kilic, A., Baysallar, M., 2015. The first *Klebsiella pneumoniae* isolate co-producing OXA-48 and NDM-1 in Turkey. Ann Lab Med 382–383. doi:10.3343/alm.2015.35.3.382

Köck, R., Daniels-Haardt, I., Becker, K., Mellmann, A., Friedrich, A.W., Mevius, D., Schwarz, S., Jurke, A., 2018. Carbapenem-resistant *Enterobacteriaceae* in wildlife, food-producing, and companion animals: a systematic review. Clin. Microbiol. Infect. doi:10.1016/j.cmi.2018.04.004

Kümmerer, K., 2009a. Antibiotics in the aquatic environment - a review - part I. Chemosphere 75, 417–34. doi:10.1016/j.chemosphere.2008.11.086

Kümmerer, K., 2009b. Antibiotics in the aquatic environment - a review - part II. Chemosphere 75, 435–441. doi:10.1016/j.chemosphere.2008.12.006

Kuwabara, B.Y.S., Abraham, E.P., 1969. Some properties of two extracellular β-lactamases from *Bacillus cereus* 569/H. biochem.J. 115, 859–861.

Larsson, D.G.G.J., de Pedro, C., Paxeus, N., 2007. Effluent from drug manufactures contains extremely high levels of pharmaceuticals. J. Hazard. Mater. 148, 751–755. doi:10.1016/j.jhazmat.2007.07.008

Larsson, D.G.J., 2014. Pollution from drug manufacturing: Review and perspectives. Philos. Trans. R. Soc. B Biol. Sci. 369. doi:10.1098/rstb.2013.0571

Laxminarayan, R., 2014. Antibiotic effectiveness: Balancing conservation against innovation. Science. 345, 1299–1301. doi:10.1126/science.1254163

Leung, M.H.Y., Lee, P.K.H., 2016. The roles of the outdoors and occupants in contributing to a potential pan-microbiome of the built environment: A review. Microbiome 4, 21. doi:10.1186/s40168-016-0165-2

Li, X.-Z., Nikaido, H., 2004. Efflux-Mediated Drug Resistance in Bacteria. [Review]. Drugs 2004 64, 159–204.

Liu, Y.-Y., Wang, Y., Walsh, T.R., Yi, L.-X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., Huang, X., Yu, L.-F., Gu, D., Ren, H., Chen, X., Lv, L., He, D., Zhou, H., Liang, Z., Liu, J.-H., Shen, J., 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet. Infect. Dis. 16, 161–8. doi:10.1016/S1473-3099(15)00424-7

Livermore, D.M., 2008. Defining an extended-spectrum β-lactamase. Clin. Microbiol. Infect. doi:10.1111/j.1469-0691.2007.01857.x

Livermore, D.M., 1992. Interplay of impermeability and chromosomal β-lactamase activity in imipenem-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 36, 2046–2048. doi:10.1128/AAC.36.9.2046

Livermore, D.M., Canton, R., Gniadkowski, M., Nordmann, P., Rossolini, G.M., Arlet, G., Ayala, J., Coque, T.M., Kern-Zdanowicz, I., Luzzaro, F., Poirel, L., Woodford, N., 2007. CTX-M: Changing the face of ESBLs in Europe. J. Antimicrob. Chemother. doi:10.1093/jac/dkl483

Machado, E., Coque, T.M., Cantón, R., Baquero, F., Sousa, J.C., Peixe, L., 2006. Dissemination in Portugal of CTX-M-15-, OXA-1-, and TEM-1-producing *Enterobacteriaceae* strains containing the *aac(6')-Ib-cr* gene, which encodes an aminoglycoside- and fluoroquinolone-modifying enzyme. Antimicrob. Agents Chemother. doi:10.1128/AAC.00473-06

Machado, E., Coque, T.M., Cantón, R., Sousa, J.C., Peixe, L., 2004. Emergence of CTX-M β-lactamase-producing *Enterobacteriaceae* in Portugal: Report of an *Escherichia coli* isolate harbouring $bla_{\text{CTX-M-14}}$. Clin. Microbiol. Infect. 10, 755–757. doi:10.1111/j.1469-0691.2004.00930.x

Magiorakos, A.P., Burns, K., Rodríguez Baño, J., Borg, M., Daikos, G., Dumpis, U., Lucet, J.C., Moro, M.L., Tacconelli, E., Simonsen, G.S., Szilágyi, E., Voss, A., Weber, J.T., 2017. Infection prevention and control measures and tools for the prevention of entry of carbapenem-resistant *Enterobacteriaceae* into healthcare settings: Guidance from the European Centre for Disease Prevention and Control. Antimicrob. Resist. Infect. Control 6, 113. doi:10.1186/s13756-017-0259-z

Mak, S., Xu, Y., Nodwell, J.R., 2014. The expression of antibiotic resistance genes in antibiotic-producing bacteria. Mol. Microbiol. 93, 391–402. doi:10.1111/mmi.12689

Manageiro, V., Jones-Dias, D., Ferreira, E., Louro, D., Caniça, M., 2012. Genetic diversity and clonal evolution of carbapenem-resistant *Acinetobacter baumannii* isolates from Portugal and the dissemination of ST118. Int. J. Antimicrob. Agents 40, 398–403. doi:10.1016/j.ijantimicag.2012.06.013

Manageiro, V., Sampaio, D.A., Pereira, P., Rodrigues, P., Vieira, L., Palos, C., Caniça, M., 2015. Draft genome sequence of the first NDM-1-producing *Providencia stuartii* strain isolated in Portugal. Genome Announc. 3, e01077-

15. doi:10.1128/genomeA.01077-15

Manaia, C.M., Macedo, G., Fatta-Kassinos, D., Nunes, O.C., 2016. Antibiotic resistance in urban aquatic environments: can it be controlled? Appl. Microbiol. Biotechnol. 100, 1543–1557. doi:10.1007/s00253-015-7202-0

Marathe, N.P., Berglund, F., Razavi, M., Pal, C., Dröge, J., Samant, S., Kristiansson, E., Larsson, D.G.J., 2019. Sewage effluent from an Indian hospital harbors novel carbapenemases and integron-borne antibiotic resistance genes. Microbiome 7, 1–11.

Marti, E., Variatza, E., Balcazar, J.L., 2014. The role of aquatic ecosystems as reservoirs of antibiotic resistance. Trends Microbiol. 22, 36–41. doi:10.1016/j.tim.2013.11.001

Marti, R., Scott, A., Tien, Y.-C., Murray, R., Sabourin, L., Zhang, Y., Topp, E., 2013. Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. Appl. Environ. Microbiol. 79, 5701–5709. doi:10.1128/AEM.01682-13

Mathlouthi, N., Areig, Z., Al Bayssari, C., Bakour, S., El Salabi, A.A., Ben Gwierif, S., Zorgani, A.A., Ben Slama, K., Chouchani, C., Rolain, J.M., 2015. Emergence of carbapenem-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* clinical isolates collected from some Libyan hospitals. Microb. Drug Resist. 21, 335–341. doi:10.1089/mdr.2014.0235

Mazar, Y., Cytryn, E., Erel, Y., Rudich, Y., 2016. Effect of dust storms on the atmospheric microbiome in the Eastern Mediterranean. Environ. Sci. Technol. 50, 4194–4202. doi:10.1021/acs.est.5b06348

Meunier, D., Doumith, M., Findlay, J., Mustafa, N., Mallard, K., Anson, J., Panagea, S., Pike, R., Wright, L., Woodford, N., Hopkins, K.L., 2016. Carbapenem resistance mediated by $bla_{OXA-181}$ in *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. doi:10.1093/jac/dkw087

Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review. Water Res. 47, 957–995. doi:10.1016/j.watres.2012.11.027

Moura, A., Soares, M., Pereira, C., Leitão, N., Henriques, I., Correia, A., 2009. INTEGRALL: A database and search engine for integrons, integrases and

gene cassettes. Bioinformatics 25, 1096–1098. doi:10.1093/bioinformatics/btp105

Naas, T., Bogaerts, P., Bauraing, C., Degheldre, Y., Glupczynski, Y., Nordmann, P., 2006. Emergence of PER and VEB extended-spectrum β-lactamases in *Acinetobacter baumannii* in Belgium. J. Antimicrob. Chemother. 58, 178–182. doi:10.1093/jac/dkl178

Naas, T., Poirel, L., Nordmann, P., 2008. Minor extended-spectrum β-lactamases. Clin. Microbiol. Infect. 14, 42–52. doi:10.1111/j.1469-0691.2007.01861.x

NCBI, 2016. Bacterial Antimicrobial Resistance Reference Gene Database [WWW Document]. URL https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047 (accessed 4.12.20).

Nicolas, I., Bordeau, V., Bondon, A., Baudy-floc, M., Felden, B., 2019. Novel antibiotics effective against Gram-positive and negative multi-resistant bacteria with limited resistance 1–23. doi:10.1371/journal.pbio.3000337

Nordmann, P., Naas, T., Poirel, L., 2011. Global spread of carbapenemase producing *Enterobacteriaceae*. Emerg. Infect. Dis. 17, 1791–1798. doi:10.3201/eid1710.110655

Nordmann, P., Poirel, L., 2016. Plasmid-mediated colistin resistance: an additional antibiotic resistance menace. Clin. Microbiol. Infect. 22, 398–400. doi:10.1016/j.cmi.2016.03.009

Nordmann, P., Poirel, L., 2014. The difficult-to-control spread of carbapenemase producers among *Enterobacteriaceae* worldwide. Clin. Microbiol. Infect. 20, 821–830. doi:10.1111/1469-0691.12719

Noval, M., Banoub, M., Claeys, K.C., Heil, E., 2020. The Battle Is on: New beta-lactams for the treatment of multidrug-resistant Gram-negative organisms. Curr. Infect. Dis. Rep. 22, 1–9.

O'Flaherty, E., Cummins, E., 2017. Antibiotic resistance in surface water ecosystems: Presence in the aquatic environment, prevention strategies, and risk assessment. Hum. Ecol. Risk Assess. An Int. J. 23, 299–322. doi:10.1080/10807039.2016.1247254

O'flaherty, E., Solimini, A.G., Pantanella, F., De Giusti, M., Cummins, E., 2018. Human exposure to antibiotic resistant-Escherichia coli through irrigated lettuce. Environ. Int. 1–11. doi:10.1016/j.envint.2018.11.022

Oliveira, C., Amador, P., Prudêncio, C., Tomaz, C.T., Tavares-Ratado, P., Fernandes, R., 2019. ESBL and AmpC β-lactamases in clinical strains of *Escherichia coli* from Serra da Estrela, Portugal. Medicina. 55, 1–13. doi:10.3390/medicina55060272

Oteo, J., Delgado-Iribarren, A., Vega, D., Bautista, V., Rodríguez, M.C., Velasco, M., Saavedra, J.M., Pérez-Vázquez, M., García-Cobos, S., Martínez-Martínez, L., Campos, J., 2008. Emergence of imipenem resistance in clinical *Escherichia coli* during therapy. Int. J. Antimicrob. Agents 32, 534–537. doi:10.1016/j.ijantimicag.2008.06.012

Ouertani, R., Limelette, A., Guillard, T., Brasme, L., Jridi, Y., Barguellil, F., El Salabi, A., De Champs, C., Chouchani, C., 2016. First report of nosocomial infection caused by *Klebsiella pneumoniae* ST147 producing OXA-48 and VEB-8 β-lactamases in Tunisia. J. Glob. Antimicrob. Resist. 4, 53–56. doi:10.1016/j.jgar.2015.10.002

Pal, C., Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2016. The structure and diversity of human, animal and environmental resistomes. Microbiome 4, 54. doi:10.1186/s40168-016-0199-5

Pärnänen, K.M.M., Narciso-Da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T.T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J.L., Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sørum, H., Stedtfeld, R.D., Tiedje, J.M., Giustina, S.V. Della, Walsh, F., Vaz-Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv. 5. doi:10.1126/sciadv.aau9124

Patel, G., Bonomo, R.A., 2013. "Stormy waters ahead": Global emergence of carbapenemases. Front. Microbiol. doi:10.3389/fmicb.2013.00048

Paterson, D.L., Bonomo, R.A., 2005. Extended-spectrum β-lactamases: A clinical update. Clin. Microbiol. Rev. doi:10.1128/CMR.18.4.657-686.2005

Peterson, E., Kaur, P., 2018. Antibiotic resistance mechanisms in bacteria: Relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. Front. Microbiol. 9, 2928. doi:10.3389/fmicb.2018.02928

Poeta, P., Radhouani, H., Igrejas, G., Goncalves, A., Carvalho, C., Rodrigues, J., Vinue, L., Somalo, S., Torres, C., 2008. Seagulls of the Berlengas Natural

Reserve of Portugal as carriers of fecal *Escherichia coli* harboring CTX-M and TEM extended-spectrum beta-lactamases. Appl. Environ. Microbiol. 74, 7439–7441. doi:10.1128/AEM.00949-08

Poirel, L., Héritier, C., Nordmann, P., 2004. Chromosome-encoded Ambler Class D β-lactamase of *Shewanella oneidensis* as a progenitor of carbapenem-hydrolyzing oxacillinase. Antimicrob. Agents Chemother. 48, 348–351. doi:10.1128/AAC.48.1.348-351.2004

Poirel, L., Héritier, C., Podglajen, I., Sougakoff, W., Gutmann, L., Nordmann, P., 2003a. Emergence in *Klebsiella pneumoniae* of a chromosome-encoded SHV β-lactamase that compromises the efficacy of imipenem. Antimicrob. Agents Chemother. 47, 755–758. doi:10.1128/AAC.47.2.755-758.2003

Poirel, L., Kämpfer, P., Nordmann, P., 2002. Chromosome-encoded ambler class A β-lactamase of Kluyvera georgiana, a probable progenitor of a subgroup of CTX-M extended-spectrum β-lactamases. Antimicrob. Agents Chemother. 46, 4038–4040. doi:10.1128/AAC.46.12.4038-4040.2002

Poirel, L., Kieffer, N., Liassine, N., Thanh, D., Nordmann, P., 2016. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*. Lancet Infect. Dis. 16, 281. doi:10.1016/S1473-3099(16)00006-2

Poirel, L., Lagrutta, E., Taylor, P., Pham, J., Nordmann, P., 2010. Emergence of metallo-β-lactamase NDM-1-producing multidrug-resistant *Escherichia coli* in Australia. Antimicrob. Agents Chemother. doi:10.1128/AAC.00878-10

Poirel, L., Liard, A., Nordmann, P., Mammeri, H., 2005. Origin of plasmid-mediated quinolone resistance determinant QnrA. Antimicrob. Agents Chemother. 49, 3523–3525. doi:10.1128/AAC.49.8.3523

Poirel, L., Menuteau, O., Agoli, N., Cattoen, C., Nordmann, P., 2003b. Outbreak of extended-spectrum β-lactamase VEB-1-producing isolates of *Acinetobacter baumannii* in a French hospital. J. Clin. Microbiol. 41, 3542–3547. doi:10.1128/JCM.41.8.3542-3547.2003

Poirel, L., Naas, T., Guibert, M., Chaibi, E.B., Labia, R., Nordmann, P., 1999. Molecular and biochemical characterization of VEB-1, a novel class A extended-spectrum β-lactamase encoded by an *Escherichia coli* integron gene. Antimicrob. Agents Chemother. 43, 573–581. doi:10.1128/aac.43.3.573

Poirel, L., Nordmann, P., 2006. Carbapenem resistance in *Acinetobacter baumannii*: Mechanisms and epidemiology. Clin. Microbiol. Infect. doi:10.1111/j.1469-0691.2006.01456.x

Poirel, L., Potron, A., Nordmann, P., 2012. OXA-48-like carbapenemases: the phantom menace. J. Antimicrob. Chemother. 67, 1597–1606. doi:10.1093/jac/dks121

Poirel, L., Ros, A., Carricajo, A., Berthelot, P., Pozzetto, B., Bernabeu, S., Nordmann, P., 2011. Extremely drug-resistant *Citrobacter freundii* isolate producing NDM-1 and other carbapenemases identified in a patient returning from India. Antimicrob. Agents Chemother. 55, 447–448. doi:10.1128/AAC.01305-10

Poole, K., 2004. Efflux-mediated multiresistance in Gram-negative bacteria. Clin. Microbiol. Infect. doi:10.1111/j.1469-0691.2004.00763.x

Potron, A., Nordmann, P., Lafeuille, E., Al Maskari, Z., Al Rashdi, F., Poirel, L., 2011a. Characterization of OXA-181, a carbapenem-hydrolyzing class D β-lactamase from *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. 55, 4896–4899. doi:10.1128/AAC.00481-11

Potron, A., Poirel, L., Bussy, F., Nordmann, P., 2011b. Occurrence of the carbapenem-hydrolyzing β-lactamase gene bla_{OXA-48} in the environment in Morocco. Antimicrob. Agents Chemother. 55, 5413–5414. doi:10.1128/AAC.05120-11

Potron, A., Poirel, L., Elhag, K., Yaqoubi, F. Al, Nordmann, P., 2009. VEB-6 extended-spectrum β-lactamase-producing *Proteus mirabilis* from sultanate of Oman. Int. J. Antimicrob. Agents 34, 493–494. doi:10.1016/j.ijantimicag.2009.05.002

Potron, A., Poirel, L., Rondinaud, E., Nordmann, P., 2013. Intercontinental spread of OXA-48 beta-lactamase-producing *Enterobacteriaceae* over a 11-year period, 2001 to 2011. Eurosurveillance 18. doi:10.2807/1560-7917.ES2013.18.31.20549

Potter, R.F., D'Souza, A.W., Dantas, G., 2016. The rapid spread of carbapenem-resistant *Enterobacteriaceae*. Drug Resist. Updat. 29, 30–46. doi:10.1016/j.drup.2016.09.002

Pulss, S., Semmler, T., Prenger-Berninghoff, E., Bauerfeind R., Ewers, C., 2017. First report of an Escherichia coli strain from swine carrying an OXA-181-

carbapenemase and colistin resistance determinant MCR-1. Int. J. Antimicrob. Agents 50, 232–236. doi:10.1016/j.ijantimicag.2017.03.014

Quale, J., Bratu, S., Gupta, J., Landman, D., 2006. Interplay of efflux system, *ampC*, and *oprD* expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical isolates. Antimicrob. Agents Chemother. 50, 1633–1641. doi:10.1128/AAC.50.5.1633-1641.2006

Queenan, A.M., Bush, K., 2007. Carbapenemases: The versatile β-lactamases. Clin. Microbiol. Rev. 20, 440–458. doi:10.1128/CMR.00001-07

Quinteira, S., Peixe, L., 2006. Multiniche screening reveals the clinically relevant metallo-β-lactamase VIM-2 in *Pseudomonas aeruginosa* far from the hospital setting: An ongoing dispersion process? Appl. Environ. Microbiol. 72, 3743–3745. doi:10.1128/AEM.72.5.3743-3745.2006

Quinteira, S., Sousa, J.C., Peixe, L., 2005. Characterization of In*100*, a new integron carrying a metallo-β-lactamase and a carbenicillinase, from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 49, 451–453. doi:10.1128/AAC.49.1.451-453.2005

Ribeiro, T.G., Novais, Â., Branquinho, R., Machado, E., Peixe, L., 2015. Phylogeny and comparative genomics unveil independent diversification trajectories of *qnrB* and genetic platforms within particular *Citrobacter* species. Antimicrob. Agents Chemother. 59, 5951–5958. doi:10.1128/AAC.00027-15

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. 447, 345–360. doi:10.1016/j.scitotenv.2013.01.032

Rogers, B.A., Sidjabat, H.E., Paterson, D.L., 2011. *Escherichia coli* O25b-ST131: A pandemic, multiresistant, community-associated strain. J. Antimicrob. Chemother. 66, 1–14. doi:10.1093/jac/dkq415

Saavedra, M.J., Peixe, L., Sousa, J.C., Henriques, I., Alves, A., Correia, A., 2003. Sfh-I, a subclass B2 metallo-β-lactamase from a *Serratia fonticola* environmental isolate. Antimicrob. Agents Chemother. 47, 2330–2333. doi:10.1128/AAC.47.7.2330-2333.2003

Saino, Y., Kobayashi, F., Inoue, M., Mitsuhashi, S., 1982. Purification and properties of inducible penicillin β-lactamase isolated from *Pseudomonas maltophilia*. Antimicrob. Agents Chemother. doi:10.1128/AAC.22.4.564

Santos, C., Ramalheira, E., Da Silva, G., Mendo, S., 2017. Genetically unrelated multidrug- and carbapenem-resistant *Citrobacter freundii* detected in outpatients admitted to a Portuguese hospital. J. Glob. Antimicrob. Resist. 8, 18–22. doi:10.1016/j.jgar.2016.09.010

Segawa, T., Takeuchi, N., Rivera, A., Yamada, A., Yoshimura, Y., Barcaza, G., Shinbori, K., Motoyama, H., Kohshima, S., Ushida, K., 2013. Distribution of antibiotic resistance genes in glacier environments. Environ. Microbiol. Rep. 5, 127–134. doi:10.1111/1758-2229.12011

Silva, I., Tacão, M., Tavares, R.D.S., Miranda, R., Araújo, S., Manaia, C.M., Henriques, I., 2018. Fate of cefotaxime-resistant *Enterobacteriaceae* and ESBL-producers over a full-scale wastewater treatment process with UV disinfection. Sci. Total Environ. 639, 1028–1037. doi:10.1016/j.scitotenv.2018.05.229

Söderström, A., Osterberg, P., Lindqvist, A., Jönsson, B., Lindberg, A., Blide Ulander, S., Welinder-Olsson, C., Löfdahl, S., Kaijser, B., De Jong, B., Kühlmann-Berenzon, S., Boqvist, S., Eriksson, E., Szanto, E., Andersson, S., Allestam, G., Hedenström, I., Ledet Muller, L., Andersson, Y., 2008. A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. Foodborne Pathog. Dis. 5, 339–49. doi:10.1089/fpd.2007.0065

Soucy, S.M., Huang, J., Gogarten, J.P., 2015. Horizontal gene transfer: Building the web of life. Nat. Rev. Genet. doi:10.1038/nrg3962

Studemeister, A.E., Quinn, J.P., 1988. Selective imipenem resistance in *Pseudomonas aeruginosa* associated with diminished outer membrane permeability. Antimicrob. Agents Chemother. doi:10.1128/AAC.32.8.1267

Surette, M.D., Wright, G.D., 2017. Lessons from the environmental antibiotic resistome. Annu. Rev. Microbiol. 71, 309–329. doi:10.1146/annurev-micro-090816-093420

Szmolka, A., Nagy, B., 2013. Multidrug resistant commensal *Escherichia coli* in animals and its impact for public health. Front. Microbiol. 4, 1–13. doi:10.3389/fmicb.2013.00258

Tacão, M., Araújo, S., Vendas, M., Alves, A., Henriques, I., 2018. Shewanella species as the origin of *bla*O_{XA-48} genes: insights into gene diversity, associated phenotypes and possible transfer mechanisms. Int. J. Antimicrob. Agents 51, 340–348. doi:10.1016/j.ijantimicag.2017.05.014

Tacão, M., Correia, A., Henriques, I., 2012. Resistance to broad-spectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of *bla*$_{CTX-M}$-like genes. Appl. Environ. Microbiol. 78, 4134–4140. doi:10.1128/AEM.00359-12

Tacão, M., Moura, A., Correia, A., Henriques, I., 2014. Co-resistance to different classes of antibiotics among ESBL-producers from aquatic systems. Water Res. 48, 100–107. doi:10.1016/j.watres.2013.09.021

Tacão, M., Tavares, R. dos S., Teixeira, P., Roxo, I., Ramalheira, E., Ferreira, S., Henriques, I., 2017. *mcr-1* and *bla*$_{kpc-3}$ in *Escherichia coli* sequence type 744 after meropenem and colistin therapy, Portugal. Emerg. Infect. Dis. doi:10.3201/eid2308.170162

Tanner, W.D., VanDerslice, J.A., Toor, D., Benson, L.S., Porucznik, C.A., Goel, R.K., Atkinson, R.M., 2015. Development and field evaluation of a method for detecting carbapenem-resistant bacteria in drinking water. Syst. Appl. Microbiol. 38, 351–357. doi:10.1016/j.syapm.2015.03.010

Teixeira, P., Tacão, M., Pureza, L., Gonçalves, J., Silva, A., Cruz-Schneider, M.P., Henriques, I., 2020. Occurrence of carbapenemase-producing *Enterobacteriaceae* in a Portuguese river: *bla*$_{NDM}$, *bla*$_{KPC}$ and *bla*$_{GES}$ among the detected genes. Environ. Pollut. 260. doi:10.1016/j.envpol.2020.113913

Topp, E., Larsson, D.G.J., Miller, D.N., Van den Eede, C., Virta, M.P.J., 2017. Antimicrobial resistance and the environment: assessment of advances, gaps and recommendations for agriculture, aquaculture and pharmaceutical manufacturing. FEMS Microbiol. Ecol. 94. doi:10.1093/femsec/fix185

Trade and Agriculture Directorate, Committee for Agriculture, 2019. Working party on agricultural policies and markets: Antibiotic use and antibiotic resistance in food producing animals in China TAD/CA/APM/WP(2018)19/FINAL. Organisation for Economic Co-operation and Development Conference Centre (OECD), Paris.

Tyers, M., Wright, G.D., 2019. Drug combinations: a strategy to extend the life of antibiotics in the 21st century. Nat. Rev. Microbiol. 17, 141–155. doi:10.1038/s41579-018-0141-x

Uppsala University, 2005. ReAct [WWW Document]. URL https://www.reactgroup.org/antibiotic-resistance/ (accessed 9.20.12).

Vahaboglu, H., Hall, L.M.C., Mulazimoglu, L., Dodanli, S., Yildirim, I., Livermore,

D.M., 1995. Resistance to extended-spectrum cephalosporins, caused by PER-1 β-lactamase, in *Salmonella typhimurium* from Istanbul, Turkey. J. Med. Microbiol. 43, 294–299. doi:10.1099/00222615-43-4-294

Valverde, A., Cantón, R., Garcillán-Barcia, M.P., Novais, Â., Galán, J.C., Alvarado, A., De La Cruz, F., Baquero, F., Coque, T.M., 2009. Spread of $bla_{CTX\text{-}M\text{-}14}$ is driven mainly by IncK plasmids disseminated among *Escherichia coli* phylogroups A, B1, and D in Spain. Antimicrob. Agents Chemother. 53, 5204–5212. doi:10.1128/AAC.01706-08

van Duin, D., Doi, Y., 2017. The global epidemiology of carbapenemase-producing *Enterobacteriaceae*. Virulence 8, 460–469. doi:10.1080/21505594.2016.1222343

van Duin, D., Kaye, K.S., Neuner, E.A., Bonomo, R.A., 2013. Carbapenem-resistant *Enterobacteriaceae*: a review of treatment and outcomes. Diagn. Microbiol. Infect. Dis. 75, 115–120. doi:10.1016/j.diagmicrobio.2012.11.009

Von Wintersdorff, C.J.H., Penders, J., Van Niekerk, J.M., Mills, N.D., Majumder, S., Van Alphen, L.B., Savelkoul, P.H.M., Wolffs, P.F.G., 2016. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Front. Microbiol. 7, 173. doi:10.3389/fmicb.2016.00173

World Health Organization (WHO), 2018. Global Health Estimates 2016: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2016.

World Health Organization (WHO), 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. doi:10.1016/S1473-3099(09)70222-1

World Health Organization (WHO) - Regional Committee for Europe, 2011. European strategic action plan on antibiotic resistance. EUR/RC61/14.

World Health Organization (WHO) - Regional Office for Europe, 2011. Tackling antibiotic resistance from a food safety perspective in Europe.

Woodford, N., Turton, J.F., Livermore, D.M., 2011. Multiresistant Gram-negative bacteria: The role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol. Rev. 35, 736–755. doi:10.1111/j.1574-6976.2011.00268.x

Wright, G.D., 2007. The antibiotic resistome: the nexus of chemical and genetic diversity. 5, 175–186. doi:10.1038/nrmicro1614

Wu, Linwei, Ning, D., Zhang, B., Li, Y., Zhang, P., Shan, X., Zhang, Q., Brown, M., Li, Z., Van Nostrand, J.D., Ling, F., Xiao, N., Zhang, Ya, Vierheilig, J., Wells, G.F., Yang, Y., Deng, Y., Tu, Q., Wang, A., Global Water Microbiome Consortium, Zhang, T., , He, Z., Keller, J., Nielsen, P.H., Alvarez, P.J.J., Criddle, C.S., Wagner, M., Tiedje, J.M., He, Q., Curtis, T.P., Stahl, D.A., Alvarez-Cohen, L., Rittmann, B.E., Wen, X., Zhou, J., 2019. Global diversity and biogeography of bacterial communities in wastewater treatment plants. Nat. Microbiol. 4, 1183–1195. doi:10.1038/s41564-019-0426-5

Yong, D., Shin, J.H., Kim, S., Lim, Y., Yum, J.H., Lee, K., Chong, Y., Bauernfeind, A., 2003. High prevalence of PER-1 extended-spectrum β-lactamase-producing *Acinetobacter* spp. in Korea. Antimicrob. Agents Chemother. 47, 1749–1751. doi:10.1128/AAC.47.5.1749-1751.2003

Yong, D., Toleman, M.A., Giske, C.G., Cho, H.S., Sundman, K., Lee, K., Walsh, T.R., 2009. Characterization of a new metallo-β-lactamase gene, *bla*$_{\text{NDM-1}}$, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob. Agents Chemother. 53, 5046–5054. doi:10.1128/AAC.00774-09

Zaman, S. Bin, Hussain, M.A., Nye, R., Mehta, V., Mamun, K.T., Hossain, N., 2017. A review on antibiotic resistance: Alarm bells are ringing. Cureus 9, e1403. doi:10.7759/cureus.1403

Zhang, C., Qiu, S., Wang, Y., Qi, L., Hao, R., Liu, X., Shi, Y., Hu, X., An, D., Li, Z., Li, P., Wang, L., Cui, J., Wang, P., Huang, L., Klena, J.D., Song, H., 2013. Higher isolation of NDM-1 producing *Acinetobacter baumannii* from the sewage of the hospitals in Beijing. PLoS One 8, e64857. doi:10.1371/journal.pone.0064857

Zhao, W.H., Hu, Z.Q., 2013. Epidemiology and genetics of CTX-M extended-spectrum β-lactamases in Gram-negative bacteria. Crit. Rev. Microbiol. doi:10.3109/1040841X.2012.691460

1.2. SCOPE OF THE WORK

Urbanization, climate change and land use alterations are recognized as fuel for the shifts and loss of biodiversity observed every day. The population growth, and consequent increase in the demand for food and water is the triggering factor to these events happen. The impact of these changes on the emergence and dispersion of pathogenic microorganisms has been investigated in detail over the past decades.

Climate change is the number one factor that is accelerating the world dynamics by changing precipitation and seasonal patterns, and increasing, recurrent and severe weather events. Natural disasters and high-impact events seen and felt as *natural* occurrences are nowadays exacerbated by human actions. Deforestation and habitat destruction, agribusiness, global trade, migration, and likewise inappropriate food-handling, inadequate sanitary conditions and poor infection control, are just a few of the factors simultaneously happening that affect the dynamics of microbial communities and consequently of infectious agents. On top of those anthropogenic actions, social behaviors and cultural habits, such as eating habits and socioeconomic dynamics, may boost zoonoses occurrence.

All of this contributed to today's scenario, in which, ironically, a pandemic (although viral) unfolds while this thesis is being written.

At the same time, environmental resistome was for many years underestimated, perhaps due to a false belief of security, which came from the evolution in medicine and the discovery of an arsenal of different antibiotic (classes) in the second half of the 20th century.

Research giving attention to resistance to critically important antibiotics, as those used for the treatment of serious infections, is more on focus than ever before. But more is still needed, particularly in what concerns resistance to last-resort antibiotics, such as those used to treat life-threatening infections caused by Gram-negative bacteria, since there are few remaining therapeutic options.

Based on the evidences stated above, the hypotheses outlined for this work were:

- Environmental bacteria, namely from aquatic environments, are the progenitors of ARGs relevant in clinical settings.
- Anthropogenic activities potentiate the dissemination of bacterial resistance in environmental ecosystems.

This work main aim was to explore the occurrence and dissemination of AR in the environment, particularly in ecosystems impacted by Men. Two general goals were established to test the hypotheses and achieve this aim:

- To assess the role of bacteria from aquatic systems as the origin of resistance genes relevant in clinical settings;
- To explore the impact of human actions in antibiotic resistance spread in aquatic environments.

Hereupon, specific goals were proposed for each chapter:

Chapter 1:

To confirm the role of *Shewanella* species as progenitors of bla_{OXA-48}-like genes and to identify elements possibly involved in the mobilization mechanism of these genes and in the transfer to other hosts. For this, isolates obtained from several aquatic systems were analyzed as well as genomes obtained from public databases. The presence of bla_{OXA-48}-like genes was assessed as well as the context of these genes and the presence of mobile genetic elements. The phenotypes conferred by these genes were evaluated in *Shewanella* isolates and in *E. coli* transformants.

Chapter 2:

To confirm the role of *Shewanella* species as progenitors of *qnrA*-like genes, isolates obtained from several aquatic systems were analyzed as well as genomes obtained from public databases. The diversity of *qnrA*-like genes was assessed.

Chapter 3:

To characterize *Escherichia coli* present in irrigation water and vegetables from household farms, in order to assess the role of irrigation water as a source of contamination of antibiotic-resistant and pathogenic *E. coli* entering the food chain. For this, *E. coli* isolates were obtained from irrigation water and vegetables and characterized to determine their clonality, diversity, antibiotic resistance genotypes and phenotypes, mobile genetic elements and virulence factors.

Chapter 4:

To characterize two multidrug-resistant isolates, obtained from vegetables in Chapter 3, by whole genome sequence analysis. The antibiotic resistance determinants, tolerance to metals determinants, mobile genetic elements, virulence factors, among others, were inspected.

Chapter 5:

To analyze carbapenem-resistant bacteria in wastewater of different treatment stages in a treatment plant applying UV-C radiation. Also, to characterize carbapenem-resistant *Enterobacteriaceae* detected in raw wastewater. For this, counts were based on cultivation of bacteria in mFC medium with or without a carbapenem. Isolates were identified and characterized in terms of antibiotic resistance phenotypes and genotypes. Whole-genome sequencing was applied to representative strains.

II. RESULTS AND DISCUSSION

II.1. ENVIRONMENTAL BACTERIA AS PROGENITORS OF RELEVANT ANTIBIOTIC RESISTANCE GENES

(CHAPTER 1 and CHAPTER 2)

CHAPTER 1: *Shewanella species as the origin of bla$_{OXA-48}$ genes: insights into gene diversity, associated phenotypes and possible transfer mechanisms*

International Journal of Antimicrobial Agents, 2018
doi: 10.1016/j.ijantimicag.2017.05.014

1. ABSTRACT

Chromosome-encoded beta-lactamases of *Shewanella* spp. have been indicated as probable progenitors of bla_{OXA-48}-like genes. However, these have been detected in few *Shewanella* spp. and dissemination mechanisms are unclear. Thus, our main objective was to confirm the role of *Shewanella* species as progenitors of bla_{OXA-48}-like genes.

In silico analysis of *Shewanella* genomes was performed to detect bla_{OXA-48}-like genes and context, and 43 environmental *Shewanella* spp. were characterized. Clonal relatedness was determined by BOX-PCR. Phylogenetic affiliation was assessed by 16S rDNA and *gyrB* sequencing. Antibiotic susceptibility phenotypes were determined. The bla_{OXA-48}-like genes and genetic context were inspected by PCR, hybridization and sequence analysis. Gene variants were cloned in *Escherichia coli* and MICs were determined. *Shewanella* isolates were screened for integrons, plasmids and insertion sequences.

Analysis of *Shewanella* spp. genomes showed that putative bla_{OXA-48}-like is present in the majority and in an identical context. Isolates presenting unique BOX profiles affiliated with 11 *Shewanella* spp. bla_{OXA-48}-like genes were detected in 22 isolates from 6 species. Genes encoded enzymes identical to OXA-48, OXA-204, OXA-181, and 7 new variants differing from OXA-48 from 2 to 82 amino acids. *IS1999* was detected in 24 isolates, although not in the vicinity of bla_{OXA-48} genes. Recombinant *E. coli* strains presented altered MICs.

The presence/absence of bla_{OXA-48}-like genes was species-related. Gene variants encoded enzymes with hydrolytic spectra similar to OXA-48-like from non-shewanellae. From the mobile elements previously described in association with bla_{OXA-48}-like genes, only the *IS1999* was found in *Shewanella*, which indicates its relevance in bla_{OXA-48}-like genes transfer to other hosts.

Keywords:

Class D carbapenemases OXA-48-like; Origin; *Shewanella.*

2. INTRODUCTION

Antibiotic resistance mechanisms occur naturally in the environment, predating the antibiotic era (D'Costa et al., 2011). Most antibiotics are derived from compounds produced by environmental microorganisms. It is therefore not surprising that antibiotic producers and neighboring microorganisms have developed mechanisms of resistance (Baquero et al., 2009; D'Costa et al., 2011).

An environmental origin has been found for some clinically-relevant resistance mechanisms. These include the widely disseminated bla_{CTX-M}, which has its putative origin in *Kluyvera* spp. (Poirel et al., 2002) and *qnrB* genes in *Citrobacter* spp. (Ribeiro et al., 2015).

The carbapenemase OXA-48 was initially identified in *Klebsiella pneumoniae* in Turkey (Poirel et al., 2012). Although initially disseminated in Mediterranean countries, OXA-48 and its variants are now an example of widely disseminated carbapenemases, detected in all continents (Poirel et al., 2012). In general, these enzymes hydrolyze penicillins and beta-lactam/beta-lactamase inhibitor combinations, early cephalosporins and also carbapenems, although with lower efficiency than other carbapenemases (Patel and Bonomo, 2013; Poirel et al., 2012). There are many reports of OXA-48-like-producers also carrying an extended spectrum beta-lactamase gene, commonly $bla_{CTX-M-15}$, or other carbapenemase coding genes, such as bla_{NDM-1} (Poirel et al., 2012; Anaïs Potron et al., 2013).

Since it was first described, bla_{OXA-48}-like acquired genes have been detected in *Enterobacteriaceae* (Galler et al., 2014; Gomez et al., 2013; Poirel et al., 2012; Potron et al., 2011b; Sampaio et al., 2014), *Acinetobacter baumannii* (Mathlouthi et al., 2015) and, recently, in *Pseudomonas aeruginosa* (Meunier et al., 2016). Although most reports refer to clinical isolates, there are also reports describing OXA-48-producers in *Enterobacteriaceae* isolated from environmental compartments, such as river water (Potron et al., 2011b) and wastewater (Galler et al., 2014).

Twelve OXA-48 variants have been reported to date, differing in 1 to 5 amino acid substitutions or deletions: OXA-162, OXA-163, OXA-181, OXA-199, OXA-

204, OXA-232, OXA-244, OXA-245, OXA-247, OXA-370, OXA-405 and OXA-416 (Antonelli et al., 2015; Dortet et al., 2015; Gomez et al., 2013; Poirel et al., 2012; Sampaio et al., 2014). Although true for most, not all OXA-48-like beta-lactamases show significant activity towards carbapenems (Dortet et al., 2015; Poirel et al., 2012).

Analysis of the genetic context of bla_{OXA-48}-like genes in *Enterobacteriaceae* has shown their association with insertion sequences (ISs) as IS*Ecp1* and IS*1999*. Also, bla_{OXA-48}-like genes are usually plasmid-borne. bla_{OXA-48} gene has been linked to IncL/M plasmids and $bla_{OXA-204}$ to IncA/C plasmids (Poirel et al., 2012). $bla_{OXA-181}$ genes have been associated with IncT and IncX3 plasmids (Liu et al., 2015; Villa et al., 2013), and with *ColE*-type, which are non-conjugative, but mobilizable, plasmids (Poirel et al., 2012; Sidjabat et al., 2013). $bla_{OXA-370}$ was associated with an IncF-like plasmid (Sampaio et al., 2014).

In 2004, Poirel and co-workers suggested for the first time that bla_{OXA-48}-like genes have their origin in *Shewanella* spp. (Poirel et al., 2012). So far, the gene variants bla_{OXA-48}, $bla_{OXA-199}$, $bla_{OXA-204}$ and $bla_{OXA-416}$ have been reported in *Shewanella xiamenensis*, although some authors have identified bla_{OXA-48}-like genes in the genome sequence of other *Shewanella* spp. (Antonelli et al., 2015; Potron et al., 2011c; Tacão et al., 2013; Zong, 2012). *Shewanella* spp. (63 species described so far) are mostly identified in aquatic ecosystems. In addition, some members are increasingly being linked to cases of human infections, acquired mainly after exposure to water through professional- or leisure-related activities (Diaz and Lopez, 2015; Janda and Abbott, 2014).

Shewanella strains carrying diverse bla_{OXA-48}-like genes are expected to be present in aquatic systems. The mechanisms of mobilization of these genes to other hosts are not clarified. Thus, the aim of this study was to analyze environmental *Shewanella* spp. isolates and genomes deposited in public databases to get insights into the role of this genus as progenitor and reservoir of bla_{OXA-48}-like genes.

3. METHODS

3.1. *In silico* analysis of *Shewanella* spp. genomes and primers design

A total of 60 *Shewanella* genomes (29 draft and 23 complete) available in National Centre for Biotechnology Information (NCBI) and Pathosystems Resource Integration (PATRIC) databases were inspected for the presence of putative bla_{OXA-48}-like genes and corresponding genetic context. The bla_{OXA-48} gene and genetic context sequence (acc. n° JX644945) from *S. xiamenensis* WCJ25 (Zong, 2012) was used for similarity searches with BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against all *Shewanella* genomes available. All bla_{OXA-48}-like genes retrieved (above 75% nucleotide similarity with bla_{OXA-48}) were aligned with multiple sequence alignment online tool Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Primer design took into account conserved regions.

3.2. Bacterial isolates, clonal relatedness and phylogenetic analysis

The *Shewanella* spp. isolates (n=43) were retrieved from estuarine water (n=17; collected in May, June and September 2008) (Azevedo et al., 2012), saltmarsh plant *Halimione portulacoides* (n=12; November 2012) (Fidalgo et al., 2016), cockle (n=11; July and November 2013) (unpublished results) and river water (n=3; July 2010) (Tacão et al., 2013). Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, USA). Partial 16S rRNA gene sequencing affiliated all isolates to the genus *Shewanella*. Clonal relatedness of isolates was determined by BOX-PCR, as described previously (Tacão et al., 2012). Isolates presenting unique banding patterns were analyzed by whole 16S rRNA gene sequencing and phylogenetic analysis. In case this analysis gave ambiguous results, *gyrB* sequencing was performed, using primers and conditions as described elsewhere (see Table S1, supplemental data). PCR products were purified with DNA Clean & Concentrator (Zymo Research, USA), and used as a template in sequencing reactions. Similarity searches were performed with BLAST software against the GenBank database. 16S rRNA gene sequences were further analyzed using the EZTaxon tool (https://www.ezbiocloud.net/identify). For

species definition, cut-off values were defined as 97% and 98% for 16S rRNA and *gyrB* gene sequences, respectively.

3.3. Antibiotic susceptibility testing

Antimicrobial resistance patterns were determined by agar disc diffusion on Mueller-Hinton agar. Discs contained the following antibiotics: ampicillin (10 µg), amoxicillin (10 µg), amoxicillin/clavulanic acid (20 µg/10 µg), piperacillin (30 µg), piperacillin/tazobactam (30 µg/6 µg), ticarcillin (75 µg), ticarcillin/clavulanic acid (75 µg/10 µg), cefotaxime (5 µg), ceftazidime (10 µg), cefepime (30 µg), imipenem (10 µg), ertapenem (10 µg), meropenem (10 µg) and aztreonam (30 µg) (Oxoid, UK). After 24 h of incubation at 37 °C, organisms were classified as susceptible or resistant according to guidelines from the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2016). The Clinical and Laboratory Standards Institute guidelines (CLSI, 2015) were used for cephalotin susceptibility tests. Minimal inhibitory concentrations (MICs) were determined in Mueller-Hinton agar according to EUCAST, for amoxicillin, amoxicillin/clavulanic acid, temocillin, cefotaxime, ceftazidime, imipenem, ertapenem and meropenem (EUCAST, 2016).

3.4. Plasmid, integron and insertion sequences screening

Plasmid DNA was purified using the EZNA Plasmid Mini-kit II (Qiagen GmbH, Germany). Detection of IncA/C, IncB/O, IncF (FIA, FIB, FIC, FIIA, FrepB subgroups), IncHI1, IncHI2, IncI1-Iγ, IncK, IncL/M, IncN, IncP IncT, IncW, IncX and IncY replicons and *ColE*-type plasmids was performed by PCR as described previously (Table S1). Using genomic DNA, integrase screening was performed for *intI1* and *intI2* genes and ISs screening was performed for IS*Ecp1* and IS*1999*, with primers and conditions as shown in Table S1. The nucleotide sequence of the amplicons was determined.

3.5. Detection of bla_{OXA-48}-like genes by PCR and hybridization

The bla_{OXA-48}-like gene fragments were amplified using primers previously reported, and also two new sets (OXA-set1_fwd/rev and OXA-set2_fwd/rev) designed in this study (Table S1) with the following PCR program: initial

denaturation of 3 min at 95°C, 30 cycles of denaturation of 30 s at 94°C, annealing of 30 s at indicated temperature and extension step of 1 min at 72°C, followed by a final extension step of 10 min at 72°C. In each reaction, water was used as negative control and *S. xiamenensis* strain IR24 carrying a bla_{OXA-48} gene as positive control (Tacão et al., 2013). Results were confirmed by sequence analysis and hybridization using OXAprobe1 and OXAprobe2 probes. To obtain OXAprobe1 and OXAprobe2 probes, bla_{OXA-48}-like genes were amplified from genomic DNA of strains *S. xiamenensis* IR24 and *Shewanella baltica* ENDN9-I using primer pairs OXAset1 and OXAset2, respectively. The amplicons were labelled during PCR by incorporation of digoxygenin-11-dUTP (Roche Molecular Biochemicals, USA). Denatured DNA was transferred to nylon membranes (Hybond N +; Amersham, Germany) using a Minifold I system (Schleicher and Schuell, Germany) and then cross-linked under UV irradiation for 5 min. Hybridization was performed in 50% formamide hybridisation buffer at 42°C, overnight. Detection was performed using the DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals, USA) following manufacturer's instructions. DNA from the bacterial isolates with plasmids identified was embedded in agarose and digested with the enzyme I-*Ceu*I (BioLabs, UK). Fragments were separated by pulsed field electrophoresis (PFGE) using a CHEF-DR II system (Bio-Rad, USA), at 6.0 V/cm for 14 h at 14°C, with an increasing pulse time of 0.2–35 s and an angle of 120°. After immobilization on nylon membranes, the I-*Ceu*I fragments were hybridized with OXAprobe1, OXAprobe2 and a 16S rDNA probe, as described above.

3.6. bla_{OXA-48}-like diversity and genetic context analysis

The nucleotide sequence of bla_{OXA-48}-like gene fragments was determined (from 798 to 888 bp). A phylogenetic tree based on the respective OXA-48-like deduced amino acid sequences, together with closest matches and representative sequences retrieved from GenBank database, was constructed using software MEGA version 6 (Tamura et al., 2013). The tree was generated using the neighbor-joining method with 1000 bootstrap replicates. The genetic context of bla_{OXA-48}-like was inspected by PCR targeting previously reported genetic elements surrounding bla_{OXA-48}-like genes. These included IS*Ecp1*, IS*1999*, C15 and *lysR*, with primers and conditions as shown in Table S1.

3.7. Transferability of bla_{OXA-48}-like genes

Mating assays were performed for bla_{OXA-48}-like-positive strains carrying plasmids. The azide-resistant *E. coli* J53 and the rifampicin-resistant *E. coli* CV601 were used as recipient strains. Transconjugants were selected in Luria-Bertani agar plates (LA) supplemented with azide (100 µg/mL) or rifampicin (100 µg/mL), and ticarcillin (50 µg/mL), as described previously (Dortet et al., 2015). If transconjugants were obtained, molecular typing with BOX-PCR was performed to confirm their identity.

3.8. Cloning experiments

To compare the hydrolytic spectra of OXA-48-like variants detected, amplicons covering bla_{OXA-48}-like genes and the genetic context were amplified by PCR, using primers and conditions as described previously (Tacão et al., 2013). Amplicons were cloned in PCR-TOPO XL vector (Invitrogen, France) and transformed by electroporation in *E. coli* TOP10, following the manufacturer's instructions. The identity of the inserts was confirmed by sequencing. The MICs for amoxicillin, amoxicillin/clavulanic acid, temocillin, cefotaxime, ceftazidime, imipenem, meropenem and ertapenem were determined for one clone of each variant, following EUCAST guidelines (EUCAST, 2016).

3.9. Nucleotide sequences

Sequences were deposited in the GenBank database under the accession numbers KX2716694–KX271708 (16S rDNA and *gyrB* sequences) and KX298208–KX298222 (bla_{OXA-48}-like sequences and genetic context).

4. RESULTS AND DISCUSSION

There are increasing indications that the putative origins of relevant antibiotic resistance mechanisms reside in environmental bacteria (Patel and Bonomo, 2013; Poirel et al., 2004; Tacão et al., 2013). *Shewanella* spp. have been mostly identified in aquatic ecosystems (Janda and Abbott, 2014); however, they have

been found in diverse ecosystems, including environmental compartments presenting extreme conditions of salinity or pressure (Nealson and Scott, 2006).

4.1. *In silico* analysis of *Shewanella* spp. genomes

In silico analysis were performed with currently available *Shewanella* genomes (query performed at 01-03-2017), to identify putative open reading frames (ORFs) encoding class D beta-lactamases, and also their genetic context (Table S2, supplemental data). The 60 *Shewanella* genomes cover a total of 22 *Shewanella* species, some represented by more than one genome, but also 18 genomes of unassigned shewanellae. OXA-like encoding genes were present in the genome of the majority of *Shewanella* spp., with most presenting from 79% to 99% similarity to OXA-48. These include the ORFs present in the genomes of *S. baltica*, *S. decolorationis*, *S. halifaxensis*, *S. haliotis*, *S. oneidensis*, *S. putrefaciens*, and *S. xiamenensis*, and also in the genome of unassigned *Shewanella* spp. (Table S2, supplemental data). On the other hand, the bla_{OXA}-like present in the genomes of *S. algae*, *S. colwelliana*, *S. fidelis*, *S. loihica*, and *S. pealeana* encodes for enzymes with only 48% to 63% similarity to OXA-48.

As shown in Table S2, the bla_{OXA} flanking regions are also conserved in *Shewanella* genomes. Despite the presence or absence of a putative bla_{OXA}, C15- and LysR-like-encoding genes are present in *Shewanella* genomes. As in other bacterial genomes (Siguier et al., 2006), several ISs are present in *Shewanella* genomes, and can be accessed in the IS-finder database (IS-Finder, http://www-is.biotoul.fr/) (Romine et al., 2008; Zong, 2012). However, we have performed an *in silico* analysis to detect ISs in the vicinity of bla_{OXA-48}-like genes, but none was identified. Furthermore, we inspected for ISs similar to IS*1999* and IS*Ecp1*, which have been identified associated with bla_{OXA-48}-like in non-shewanellae strains. IS*Ecp1* was not identified in *Shewanella* genomes and IS*1999* was identified in only 4 *Shewanella* genomes (1 *S. xiamenensis*, 1 *S. putrefaciens* and 2 *Shewanella* sp.), all carrying bla_{OXA} genes (see supplemental data, Table S2). As far as we know, there is only one report on ISs in the vicinity of bla_{OXA-48}-like in *Shewanella* spp. (i.e. IS*Shes2* inserted between $bla_{OXA-199}$ and *lysR*) (Zong, 2012).

4.2. *Shewanella* isolates diversity and antibiotic susceptibility profiles

We gathered *Shewanella* isolates from four different water-related environmental compartments: saltmarsh plants, cockle, estuarine and river water. From 43 *Shewanella* isolates, 33 displayed unique BOX-PCR patterns. Unique isolates affiliated with *Shewanella hafniensis* (n=9), *S. xiamenensis* (n=5), *Shewanella aestuarii* (n=4), *S. baltica* (n=4), *S. haliotis* (n=2), *Shewanella indica* (n=2), *S. putrefaciens* (n=2), *Shewanella algidipiscicola* (n=1), *S. algae* (n=1), *Shewanella fodinae* (n=1) and *Shewanella* sp. (n=2) (Table 1).

Overall, the most frequently detected was *S. hafniensis* (27.3%), isolated in cockle and estuarine water, followed by *S. xiamenensis* (15.2%), isolated from saltmarsh plants and river water. Some of the species identified in this study are considered opportunistic human pathogens and have been implicated in clinical cases. These include *S. putrefaciens*, *S. algae* and *S. haliotis*, which have been associated with skin and soft tissue infections, and *S. xiamenensis*, which has been linked to pancreatitis (Janda, 2014a, 2014b; Janda and Abbott, 2014; Tsai et al., 2008; Zong, 2011). It has been proposed that both *S. xiamenensis* and *S. haliotis* should be listed as species of medical and public health importance (Janda, 2014b). Isolates were susceptible to most beta-lactams (Table 1). Lower resistance levels were observed towards third- and fourth-generation cephalosporins (6.1% of resistant isolates towards cefotaxime and 6.1% for ceftazidime), and much higher resistance levels were found towards the early cephalosporin, cephalotin (87.9%). Concerning carbapenems, resistance levels were lower against meropenem than for ertapenem (18.2% vs. 36.4%). *S. xiamenensis* isolates presented similar antibiotic susceptibility profiles within the species, but the same was not true for other species in the collection. For example, *S. aestuarii* Sh13 was susceptible to all antibiotics tested, whereas *S. aestuarii* Sh12 was resistant to penicillins, cefotaxime, meropenem and imipenem. Thus, antibiotic susceptibility patterns were not always species-related, which indicates the occurrence of unidentified acquired mechanisms.

TABLE 1. Bacterial strains, isolation source, antibiotic susceptibility profiles (dark grey - resistant, light grey - susceptible) and presence/absence of *bla*$_{OXA-48}$-like genes, plasmids and IS*1999* (filled circle - detected, open circle - not detected).

Strain	Source[a]	Affiliation	Antibiotic susceptibility profiles[b]															*bla*$_{OXA-48}$-like	Plasmids	IS*1999*
			AMP	AML	AMC	PIP	TZP	TIC	TIM	CEF	CTX	CAZ	FEP	ETP	MEM	IPM	ATM			
Sh1	SP	*S. xiamenensis*				■		■	■	■				■		■	■	●	●	●
Sh2	SP	*S. algae*								■								●	○	●
Sh3	SP	*S. fodinae*	■	■	■	■	■	■	■	■	■	■	■	■		■		●	○	○
Sh4	SP	*S. haliotis*	■	■					■	■						■		○	○	○
Sh5	SP	*S. xiamenensis*		■	■	■		■	■	■				■	■	■		●	○	●
Sh6	SP	*Shewanella sp.*																○	●	●
Sh7	SP	*Shewanella sp.*				■	■					■					■	○	○	●
Sh8	CL	*S. aestuarii*								■								○	●	●
Sh9	CL	*S. baltica*			■					■								●	●	●
Sh10	CL	*S. hafniensis*								■								●	○	●
Sh11	CL	*S. aestuarii*		■						■								○	○	●
Sh12	CL	*S. aestuarii*	■	■		■	■	■	■		■				■	■		○	●	●
Sh13	CL	*S. aestuarii*																○	○	●
Sh14	CL	*S. haliotis*	■	■	■	■	■	■	■	■				■		■	■	○	○	●
Sh15	CL	*S. indica*								■								○	○	●
Sh16	CL	*S. indica*	■			■				■					■			○	○	○
Sh17	EW	*S. hafniensis*								■								●	○	●
Sh18	EW	*S. hafniensis*								■								●	○	○
Sh19	EW	*S. baltica*								■				■				●	○	●
Sh20	EW	*S. hafniensis*	■	■		■				■				■				●	○	○
Sh21	EW	*S. baltica*								■				■				●	○	○
Sh22	EW	*S. baltica*								■				■				●	○	●
Sh23	EW	*S. putrefaciens*								■								●	○	●
Sh24	EW	*S. hafniensis*								■								●	○	●
Sh25	EW	*S. hafniensis*								■								●	●	●
Sh26	EW	*S. hafniensis*								■								●	●	○
Sh27	EW	*S. putrefaciens*	■	■	■			■		■				■				●	○	●
Sh28	EW	*S. algidipiscicola*								■								○	○	○
Sh29	EW	*S. hafniensis*		■	■					■								●	○	○
Sh30	EW	*S. hafniensis*								■								●	●	●
Sh31	RW	*S. xiamenensis*	■	■	■	■		■	■	■				■	■	■		●	●	●
Sh32	RW	*S. xiamenensis*	■	■	■			■	■	■				■	■	■		●	●	●
Sh33	RW	*S. xiamenensis*	■	■	■	■		■	■	■				■	■	■		●	○	●

[a]Isolation sources: SP - saltmarsh plant, CL - cockle, EW - estuarine water, RW - river water;

[b]Antibiotics: AMP - ampicillin, AMC - amoxicillin, AMC - amoxicillin/clavulanic acid, PIP - piperacillin, TZP - piperacillin/tazobactam, TIC - ticarcillin, TIM - ticarcillin/clavulanic acid, CEF - cephalotin, CTX - cefotaxime, CAZ - ceftazidime, FEP - cefepime, ETP - ertapenem, MEM - meropenem, IPM - imipenem, ATM - aztreonam.

According to other authors, shewanellae are commonly susceptible to extended-spectrum cephalosporins, beta-lactam/beta-lactamase inhibitor combinations, carbapenems and also to non-beta-lactams, such as aminoglycosides and quinolones (Janda, 2014a). Ceftazidime, ciprofloxacin or gentamicin have been widely chosen for treating most infections linked to shewanellae (Tsai et al., 2008). Nevertheless, there are sporadic reports of clinical and environmental *Shewanella* strains showing different resistance profiles, for example, reduced susceptibility to carbapenems (Antonelli et al., 2015; Tacão et al., 2013).

4.3. Occurrence, diversity and genetic context of bla_{OXA-48}-like genes

bla_{OXA-48}-like genes were detected in 22 *Shewanella* isolates: *S. hafniensis* (9 positive isolates of 9 tested isolates), *S. xiamenensis* (5/5), *S. baltica* (4/4), *S. algae* (1/1), *S. fodinae* (1/1), and *S. putrefaciens* (2/2) (Table 1). In 19 isolates, bla_{OXA-48}-like genes were identified by both PCR and hybridisation. For the two *S. putrefaciens* isolates and the single *S. algae*, bla_{OXA-48}-like genes seem to be present according to hybridization results, but were not amplified by PCR. Overall, it was possible to amplify and sequence the complete bla_{OXA-48}-like gene from 19 strains (Table 2). Genes encoded enzymes that were 100% identical to OXA-48, OXA-204 and OXA-181, and 7 new variants differing from OXA-48 from 2 to 82 amino acids (Table 2, Figure 1). From the alignment of amino acid sequences, it was possible to identify the amino acid motifs that are conserved among class D beta-lactamases (Oueslati et al., 2015; Poirel et al., 2010c), for example, Arg 214, which has been proven critical for carbapenemase activity (Dortet et al., 2015) (Figure 1).

A phylogenetic tree was constructed based on the deduced amino acid sequences of: (i) all variants identified in this work, (ii) variants previously reported in clinical isolates and also (iii) variants present in *Shewanella* genomes available in public databases (Figure 2). Variants identified in this work were included in three clusters: (i) cluster 1, which comprised OXA-48, OXA-181, OXA-204 and OXA-546 detected in this study in *S. xiamenensis*, but also all variants that have been reported in *Enterobacteriaceae* members (Antonelli et al., 2015; Dortet et al., 2015; Gomez et al., 2013; Poirel et al., 2012; Sampaio et al., 2014),

Acinetobacter baumannii (Mathlouthi et al., 2015) and, recently, in a clinical *P. aeruginosa* strain (Meunier et al., 2016); (ii) cluster 2, with variants detected in *S. hafniensis* and (iii) cluster 3, with OXA-551 and other variants identified in *S. baltica* genomes.

TABLE 2. *Shewanella* isolates carrying bla_{OXA-48}-like genes, genomic context of the gene, OXA-like variants detected and similarity to OXA-48 (% identity in terms of amino acid sequence) (filled circle - detected, open circle - not detected).

Isolates	bla_{OXA-48}-like gene detection		bla_{OXA-48}-like gene context		OXA-48-like variants		
	Hybridization	PCR	Upstream C15	Downstream *lysR*	OXA-48-like variant	Nº residues differing from OXA-48	% similarity to OXA-48
***S. xiamenensis* Sh1**	●	●	●	●	OXA-VAR1	2	99%
***S. algae* Sh2**	●	○	○	○	-	-	-
***S. fodinae* Sh3**	●	●	●	○	OXA-181	4	98%
***S. xiamenensis* Sh5**	●	●	●	●	OXA-181	4	98%
***S. baltica* Sh9**	●	●	○	●	-	-	-
***S. hafniensis* Sh10**	●	●	●	●	OXA-VAR2	81	81%
***S. hafniensis* Sh17**	●	●	●	●	OXA-VAR3	74	81%
***S. hafniensis* Sh18**	●	●	●	●	OXA-VAR3	74	81%
***S. baltica* Sh19**	●	●	●	●	OXA-VAR4	82	80%
***S. hafniensis* Sh20**	●	●	●	●	OXA-VAR5	77	80%
***S. baltica* Sh21**	●	●	●	●	OXA-VAR4	82	80%
***S. baltica* Sh22**	●	●	●	●	OXA-VAR4	82	80%
***S. putrefaciens* Sh23**	●	○	○	○	-	-	-
***S. hafniensis* Sh24**	●	●	●	●	OXA-VAR3	74	81%
***S. hafniensis* Sh25**	●	●	●	●	OXA-VAR3	74	81%
***S. hafniensis* Sh26**	●	●	●	●	OXA-VAR7	74	81%
***S. putrefaciens* Sh27**	●	○	○	○	-	-	-
***S. hafniensis* Sh29**	●	●	●	●	OXA-VAR6	76	81%
***S. hafniensis* Sh30**	●	●	●	●	OXA-VAR3	74	81%
***S. xiamenensis* Sh31**	●	●	●	●	OXA-48	-	100%
***S. xiamenensis* Sh32**	●	●	●	●	OXA-48	-	100%
***S. xiamenensis* Sh33**	●	●	●	●	OXA-204	2	99%

It is also important to notice that the same variant ($bla_{OXA-181}$) was identified in the single *S. fodinae* strain included in this study as well as in *S. xiamenensis*. Although this result may indicate horizontal gene transfer between these two species, the fact that it refers to a single *S. fodinae* limits its interpretation, as does the lack of *S. fodinae* genomes available for additional analysis.

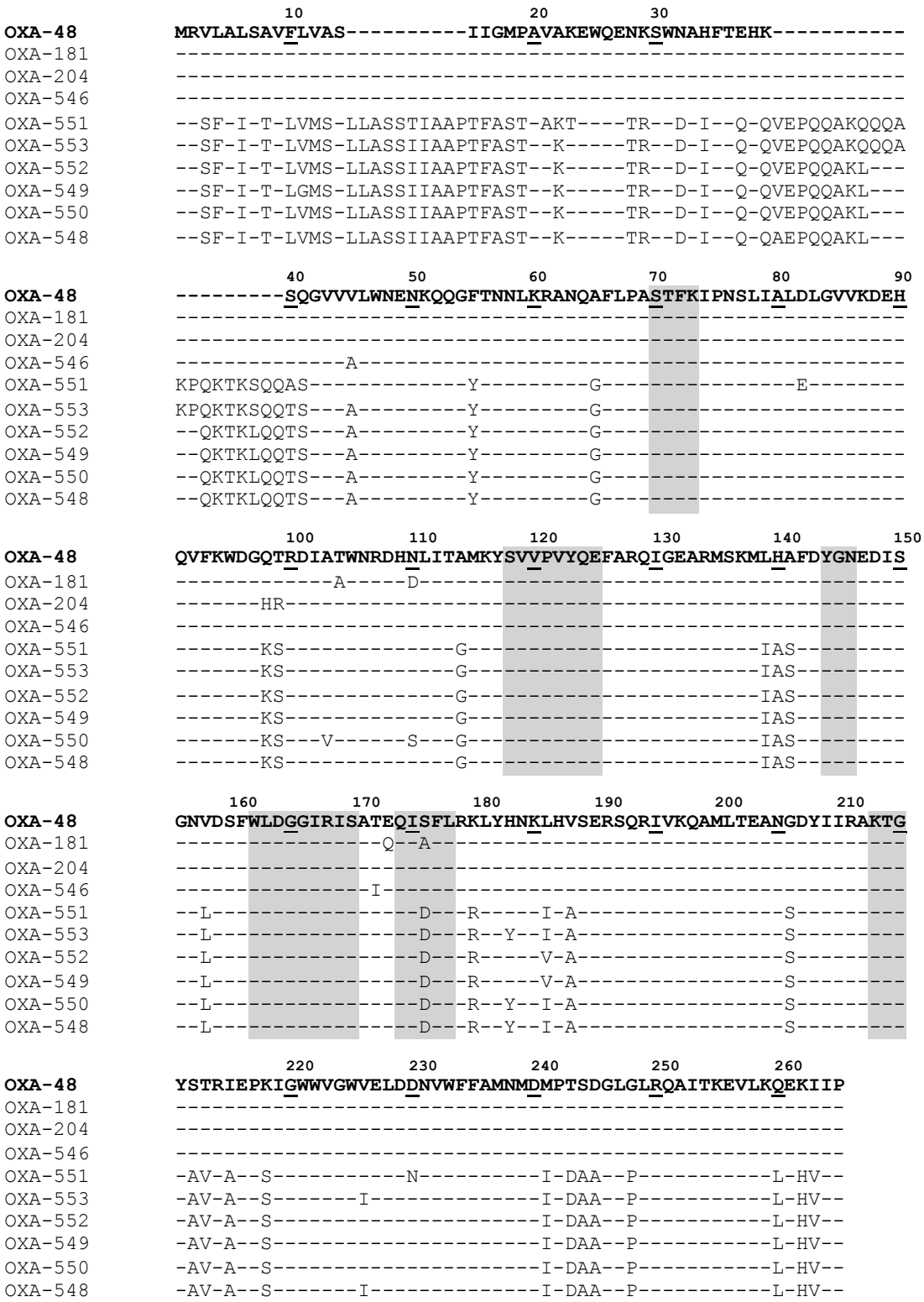


FIGURE 1. Alignment of the amino acid sequences of OXA-48, OXA-181, OXA-204, and new variants OXA-VAR1 to OXA-VAR7. Amino acid motifs that are conserved among class D beta-lactamases are indicated in grey boxes. Numbering is according to the class D beta-lactamase system (DBL) (Couture et al., 1992).

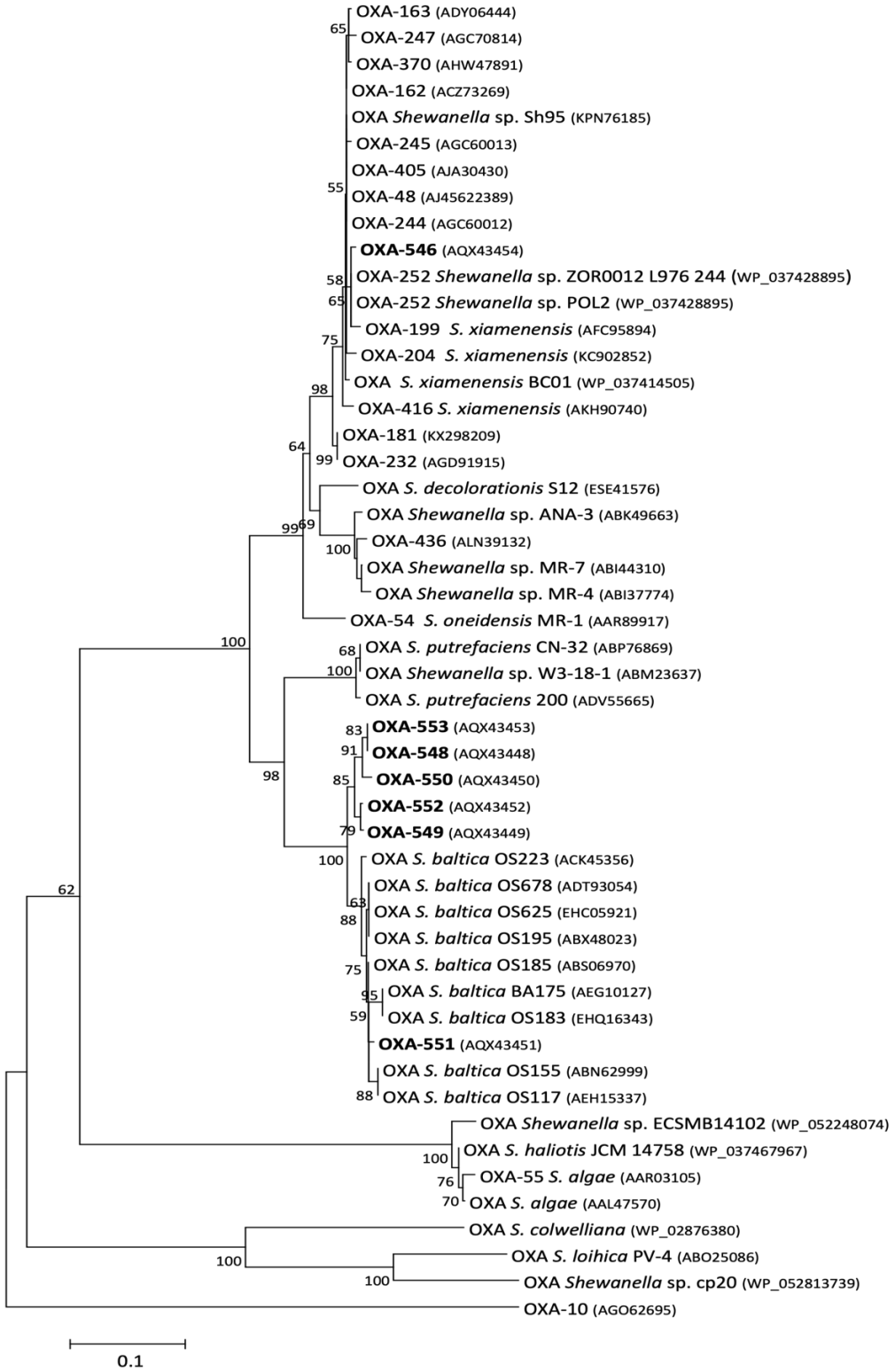


FIGURE 2. Phylogenetic tree based on OXA-48-like deduced amino acid sequences detected in *Shewanella* isolates obtained in this study (in bold) together with closest matches and representative sequences retrieved from GenBank database. The tree was generated using the neighbour-joining method tree with 1000 bootstrap replicates. Bootstrap confidence is shown in %.

Different gene variants have been associated with diverse genetic contexts in non-*Shewanella* hosts (Poirel et al., 2012), which indicates independent mobilization events, probably from different *Shewanella* strains. Although most cases concern clinical isolates, bla_{OXA-48} has also been reported in *Serratia marcescens* isolated from river water (Galler et al., 2014). As pointed out by other authors (Poirel et al., 2012; Zong, 2012), this indicates that the mobilization of bla_{OXA-48}-like genes from the *Shewanella* chromosome to clinical hosts may be direct or mediated by environmental intermediates.

In the present study, the genetic context of the bla_{OXA-48}-like genes was determined for 17 isolates (Table 2). In these cases, genetic context analysis revealed C15 gene upstream and *lysR* gene down-stream, identical to that previously reported for bla_{OXA-48}-like genes in *Shewanella* spp. (Tacão et al., 2013; Zong, 2012), and as observed through *in silico* analysis of *Shewanella* genomes (Table S2).

Overall results indicate that bla_{OXA-48}-like genes are intrinsic to each species, probably evolving from a common ancestor, and in parallel with genus evolution. Putative bla_{OXA} genes are present in the majority, but not all, of *Shewanella* spp. (Table S2). We can speculate that the ancestral gene was acquired when bla_{OXA}-carrying species had already separated from others. Another hypothesis is that *Shewanella* spp. that do not have the gene might have lost it later. Given this, our data support the hypothesis that this resistance mechanism is intrinsic to some species of *Shewanella*, such as *S. xiamenensis*, *S. hafniensis*, *S. putrefaciens* or *S. baltica*.

4.4. Integrons, plasmids and transferability of bla_{OXA-48}-like genes

Plasmids were detected in 30.3% (10 of 33) of *Shewanella* isolates and all were submitted to replicon typing. Only for the plasmid detected in *S. aestuarii*, Sh12, was it possible to assign to the replicon IncHI1. From the available *Shewanella* spp. genomes, we observe that plasmids have been recognized in *S. baltica* and *S. oneidensis*, although not allocated to any replicon typing.

Conjugation assays were performed for all isolates carrying bla_{OXA-48}-like genes and plasmids (n=7). No transconjugants were obtained. We also inspected

for mobilizable *ColE*-type plasmids that have already been associated to bla_{OXA-48}-like genes in *Enterobacteriaceae* (Sidjabat et al., 2013), but none was detected. The genetic location of bla_{OXA-48}-like was also analyzed for these isolates by I-*Ceu*I-digested genomic DNA subjected to PFGE, followed by hybridization with both 16S rDNA and OXAvar1/OXAvar2 probes. Results confirmed the chromosomal location of bla_{OXA-48}-like genes in all isolates, except for *S. hafniensis* Sh26 (see Figure S1 in supplemental material). Although the experiment was repeated, the gene location in this strain could not be confirmed. Nevertheless, considering the overall results, including the analysis of *Shewanella* available genomes, most likely the bla_{OXA-48}-like genes identified in all *Shewanella* strains are chromosomally-located, in accordance with previous suggestions (Poirel et al., 2012).

For all isolates, we performed a PCR screening for ISs previously reported in association with bla_{OXA-48}-like genes. In contrast with *in silico* results, we detected IS*1999* in 24 isolates, 17 (85%) of which were carrying bla_{OXA-48}-like genes. However, the IS was not detected in the genetic context of the bla_{OXA-48}-like genes. Similarity searches performed against the GenBank database showed that the ISs fragment was 99-100% similar to IS*1999* found in diverse genetic environments. This insertion sequence has frequently been found associated with bla_{OXA-48}-like genes in *Enterobacteriaceae* isolates (Poirel et al., 2012). Its frequent occurrence might indicate that IS*1999* plays a role in the mobilization and transfer mechanisms from *Shewanella* spp. to other hosts.

As stated above, IS*1999*-like insertion elements were identified in only 4 of 62 *Shewanella* genomes available so far. This may indicate that its acquisition might depend on the adaptation of *Shewanella* strains to a certain environment. In this perspective, we can speculate that the transfer of bla_{OXA-48}-like genes from shewanellae to other hosts is more prone to occur in specific environmental compartments, such as aquatic settings.

Although sporadic, there are reports of *Shewanella* spp. carrying class 1 and class 2 integrons, with gene cassette arrays encoding antibiotic resistance (Couture et al., 1992; Ramírez et al., 2010; Zhao et al., 2015). Here we also performed class 1 and class 2 integron screening, but no positive results were obtained.

4.5. Hydrolytic spectra of OXA-48-like variants identified

One representative of each bla_{OXA-48}-like variant identified was selected for cloning and transformation in *E. coli*. Transformants showed high-level resistance to amoxicillin, amoxicillin with clavulanic acid and temocillin (Table 3). Decreased susceptibility to carbapenems was noted for all transformants, with the majority showing this trait for the three carbapenems tested. For both imipenem and meropenem, we observed an upsurge of 2- to 8-fold, whereas for ertapenem a 2- to 60-fold increase in MICs was observed. A decrease in susceptibility was also registered (2- to 4-fold) for the third generation cephalosporins tested. These results agree with earlier reports for the majority of variants described as OXA-48-like 'true carbapenemases', considering as exceptions the variants OXA-163, OXA-247 and OXA-405, which present higher MICs towards expanded spectrum cephalosporins, such as ceftazidime and cefotaxime (Dortet et al., 2015; Oueslati et al., 2014; Poirel et al., 2012).

As highlighted previously (Oueslati et al., 2014), when comparing MICs that have been reported by different authors, we must take into consideration that cloning experiments are performed with different plasmids and expressed in different strains and, therefore, are in different genetic backgrounds. Nevertheless, it is possible to compare the overall hydrolytic features. Thus, the variants reported here generally showed similar hydrolytic spectra to that of OXA-48, and in accordance with previous reports: high MICs towards penicillins (not altered when combined with beta-lactamase inhibitors), and reduced susceptibility to at least one of the carbapenems tested (Poirel et al., 2012). To highlight this, MICs for temocillin were high, varying from 32 mg/L to over 1024 mg/L. These results are in line with the suggestion of the EUCAST subcommittee for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance that has designated high-level temocillin resistance (MIC >32 mg/L) as a phenotypic indicator of OXA-48 production (EUCAST, 2016).

Finally, it is also important to highlight that infections linked to shewanellae are increasingly being reported, particularly related to *S. xiamenensis*, *S. algae*, *S. putrefaciens* and *S. haliotis*, which all carry class D carbapenemases OXA-48-like, as shown in this study, thus disabling carbapenem use as treatment option (Poirel et al., 2012).

TABLE 3. MICs of beta-lactams for shewanellae donor strains, *E. coli* pTOPO-OXA-48, *E. coli* pTOPO-OXA-181, *E. coli* pTOPO-OXA-204, *E. coli* pTOPO-OXA-VAR1 to *E. coli* pTOPO-OXA-VAR7 and *E. coli* TOP10.

	β-lactams MIC (mg/L)[a]							
	AML	AMC	TMC	CTX	CAZ	IPM	ETP	MEM
S. xiamenensis Sh31 bla_{OXA-48}	256	96	3	0.5	0.25	8	8	2
E. coli TOP10 (pTOPO-OXA-48)	>256	>256	512	1	0.5	1	1	0.25
S. xiamenensis Sh5 $bla_{OXA-181}$	8	12	1	0.5	0.12	4	4	5
E. coli TOP10 (pTOPO-OXA-181)	>256	>256	>1024	1	0.5	2	1	0.25
S. xiamenensis Sh33 $bla_{OXA-204}$	>256	>256	8	0.25	0.25	>32	>32	8
E. coli TOP10 (pTOPO-OXA-204)	16	8	32	2	0.12	0.5	0.016	0.06
S. xiamenensis Sh1 $bla_{OXA-546}$	4	4	1	0.5	0.25	1	3	0.5
E. coli TOP10 (pTOPO-OXA-546)	>256	>256	1024	1	0.25	1	1	0.25
S. hafniensis Sh10 $bla_{OXA-553}$	2	1.5	0.19	0.25	0.03	0.12	0.038	0.3
E. coli TOP10 (pTOPO-OXA-553)	16	12	32	1	0.25	0.5	0.032	0.06
S. hafniensis Sh24 $bla_{OXA-552}$	4	1.5	0.125	0.06	0.015	0.25	0.25	0.015
E. coli TOP10 (pTOPO-OXA-552)	>256	>256	>1024	2	0.25	1	0.5	0.12
S. baltica Sh19 $bla_{OXA-551}$	16	12	0.19	0.25	12	0.5	1	0.06
E. coli TOP10 (pTOPO-OXA-551)	>256	>256	>1024	2	0.25	2	1	0.5
S. hafniensis Sh20 $bla_{OXA-550}$	16	4	3	0.5	0.06	1	0.75	0.12
E. coli TOP10 (pTOPO-OXA-550)	>256	>256	>1024	2	0.25	1	0.38	0.12
S. hafniensis Sh29 $bla_{OXA-548}$	4	2	0.125	0.12	0.015	0.25	0.25	0.12
E. coli TOP10 (pTOPO-OXA-548)	16	12	32	2	0.25	0.5	0.047	0.06
S. hafniensis Sh26 $bla_{OXA-549}$	4	1	0.19	0.06	0.015	0.25	0.25	0.03
E. coli TOP10 (pTOPO-OXA-549)	>256	>256	1024	2	0.12	0.5	0.19	0.06
***E. coli* TOP10**	**4**	**4**	**8**	**0.5**	**0.12**	**0.25**	**0.016**	**0.06**

[a]AML- amoxicillin, AMC- amoxicillin/clavulanic acid, TMC- temocillin, CTX- cefotaxime, CAZ- ceftazidime, IPM- imipenem, ETP- ertapenem, MEM- meropenem.

5. CONCLUSIONS

Overall, the results presented in this paper reinforce the role of *Shewanella* as progenitor of bla_{OXA-48}-like genes. Several new OXA-48 variants were identified in different members of this genus. Moreover, both *in silico* and molecular analysis indicate that the presence/absence of bla_{OXA-48}-like genes seems to be species-related, and thus intrinsic to some *Shewanella* species. The fact that IS*1999* was detected in the majority of isolates indicates that it might be involved in the mobilization mechanism of these genes and transfer from *Shewanella* spp. to other hosts. However, more studies are needed to confirm this hypothesis. On the other hand, we have not found any evidence of these genes associated to integrons or plasmids. We have confirmed that bla_{OXA-48}-like genes detected in different *Shewanella* strains code for enzymes that have hydrolytic spectra similar to the majority that have been described so far in non-shewanellae, which

includes carbapenems. These new variants and variants that remain unnoticed can eventually reach clinical settings and become a threat, mainly by incapacitating use of carbapenems.

6. ACKNOWLEDGMENTS

We thank Alessandra Carattoli (Department of Infectious, Parasitic and Immune-mediated Diseases at the Istituto Superiore di Sanità, Rome, Italy) for providing replicon typing control strains. The authors also wish to acknowledge COST-European Cooperation in Science and Technology, through COST Action ES1403: New and emerging challenges and opportunities in wastewater reuse (NEREUS).

Funding: This work was supported by Fundação para a Ciência e Tecnologia through project StARE (WaterJPI/0002/2013) and grants IF/00492/2013 (I. Henriques), IF/00835/2013 (A. Alves), SFRH/BPD/114855/2016 (M. Tacão) and SFRH/BD/52573/2014 (S. Araújo). Thanks are due also for the financial support to CESAM (UID/AMB/50017-POCI-01-0145-FEDER-007638), to FCT/MCTES through national funds (PIDDAC), and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020.

7. REFERENCES

Antonelli, A., Di Palo, D.M., Galano, A., Becciani, S., Montagnani, C., Pecile, P., Galli, L., Rossolini, G.M., 2015. Intestinal carriage of *Shewanella xiamenensis* simulating carriage of OXA-48-producing *Enterobacteriaceae*. Diagn. Microbiol. Infect. Dis. 82, 1–3. doi:10.1016/j.diagmicrobio.2015.02.008

Azevedo, J.S.N., Ramos, I., Araújo, S., Oliveira, C.S., Correia, A., Henriques, I.S., 2012. Spatial and temporal analysis of estuarine bacterioneuston and bacterioplankton using culture-dependent and culture-independent methodologies. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 101, 819–835. doi:10.1007/s10482-012-9697-z

Baquero, F., Alvarez-Ortega, C., Martinez, J.L., 2009. Ecology and evolution of antibiotic resistance. Environ. Microbiol. Rep. doi:10.1111/j.1758-2229.2009.00053.x

Clinical and Laboratory Standards Institute, 2015. Performance standards for antimicrobial susceptibility testing, 27th ed. ed. Wayne, PA, USA.

Couture, F., Lachapelle, J., Levesque, R.C., 1992. Phylogeny of LCR-1 and OXA-5 with class A and class D β-lactamases. Mol. Microbiol. 6, 1693–1705. doi:10.1111/j.1365-2958.1992.tb00894.x

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., Wright, G.D., 2011. Antibiotic resistance is ancient. Nature 477, 457–461. doi:10.1038/nature10388

Diaz, J.H., Lopez, F.A., 2015. Skin, soft tissue and systemic bacterial infections following aquatic injuries and exposures. Am. J. Med. Sci. 349, 269–275. doi:10.1097/MAJ.0000000000000366

Dortet, L., Oueslati, S., Jeannot, K., Tandé, D., Naas, T., Nordmann, P., 2015. Genetic and biochemical characterization of OXA-405, an OXA-48-type extended-spectrum β-lactamase without significant carbapenemase activity. Antimicrob. Agents Chemother. 59, 3823–3828. doi:10.1128/AAC.05058-14

EUCAST, 2016. Breakpoint tables for interpretation of MICs and zone diameters.

Fidalgo, C., Henriques, I., Rocha, J., Tacão, M., Alves, A., 2016. Culturable endophytic bacteria from the salt marsh plant *Halimione portulacoides*: phylogenetic diversity, functional characterization, and influence of metal(loid) contamination. Environ. Sci. Pollut. Res. 23, 10200–10214. doi:10.1007/s11356-016-6208-1

Galler, H., Feierl, G., Petternel, C., Reinthaler, F.F., Haas, D., Grisold, A.J., Luxner,

J., Zarfel, G., 2014. KPC-2 and OXA-48 carbapenemase-harbouring *Enterobacteriaceae* detected in an Austrian wastewater treatment plant. Clin. Microbiol. Infect. 20, O132–O134. doi:10.1111/1469-0691.12336

Gomez, S., Pasteran, F., Faccone, D., Bettiol, M., Veliz, O., De Belder, D., Rapoport, M., Gatti, B., Petroni, A., Corso, A., 2013. Intrapatient emergence of OXA-247: A novel carbapenemase found in a patient previously infected with OXA-163-producing *Klebsiella pneumoniae*. Clin. Microbiol. Infect. doi:10.1111/1469-0691.12142

Janda, J.M., 2014a. Shewanella: A marine pathogen as an emerging cause of human disease. Clin. Microbiol. Newsl. 36, 25–29. doi:10.1016/j.clinmicnews.2014.01.006

Janda, J.M., 2014b. Taxonomic update on enteric- and aquatic-associated Gram-negative bacteria: Proposed new species and classification changes. Clin. Microbiol. Newsl. 36, 1–5. doi:10.1016/j.clinmicnews.2013.12.001

Janda, J.M., Abbott, S.L., 2014. The genus *Shewanella*: From the briny depths below to human pathogen. Crit. Rev. Microbiol. doi:10.3109/1040841X.2012.726209

Liu, Y., Feng, Y., Wu, W., Xie, Y., Wang, X., Zhang, X., Chen, X., Zong, Z., 2015. First report of OXA-181-producing *Escherichia coli* in China and characterization of the isolate using whole-genome sequencing. Antimicrob. Agents Chemother. 59, 5022–5025. doi:10.1128/AAC.00442-15

Mathlouthi, N., Areig, Z., Al Bayssari, C., Bakour, S., El Salabi, A.A., Ben Gwierif, S., Zorgani, A.A., Ben Slama, K., Chouchani, C., Rolain, J.M., 2015. Emergence of carbapenem-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* clinical isolates collected from some Libyan hospitals. Microb. Drug Resist. 21, 335–341. doi:10.1089/mdr.2014.0235

Meunier, D., Doumith, M., Findlay, J., Mustafa, N., Mallard, K., Anson, J., Panagea, S., Pike, R., Wright, L., Woodford, N., Hopkins, K.L., 2016. Carbapenem resistance mediated by $bla_{OXA-181}$ in *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. doi:10.1093/jac/dkw087

Nealson, K.H., Scott, J., 2006. Ecophysiology of the genus *Shewanella*. The Prokaryotes. Springer New York, pp. 1133–1151. doi:10.1007/0-387-30746-x_45

Oueslati, S., Nordmann, P., Poirel, L., 2014. Heterogeneous hydrolytic features for OXA-48-like β-lactamases. J. Antimicrob. Chemother. 70, 1059–1063. doi:10.1093/jac/dku524

Patel, G., Bonomo, R.A., 2013. "Stormy waters ahead": Global emergence of carbapenemases. Front. Microbiol. doi:10.3389/fmicb.2013.00048

Poirel, L., Héritier, C., Nordmann, P., 2004. Chromosome-encoded Ambler class D β-Lactamase of *Shewanella oneidensis* as a progenitor of carbapenem-hydrolyzing oxacillinase. Antimicrob. Agents Chemother. 48, 348–351. doi:10.1128/AAC.48.1.348-351.2004

Poirel, L., Kämpfer, P., Nordmann, P., 2002. Chromosome-encoded Ambler class A β-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum β-lactamases. Antimicrob. Agents Chemother. 46, 4038–4040. doi:10.1128/AAC.46.12.4038-4040.2002

Poirel, L., Naas, T., Nordmann, P., 2010. Diversity, epidemiology, and genetics of class D beta-lactamases. Antimicrob. Agents Chemother. 54, 24–38. doi:10.1128/AAC.01512-08

Poirel, L., Potron, A., Nordmann, P., 2012. OXA-48-like carbapenemases: the phantom menace. J. Antimicrob. Chemother. 67, 1597–1606. doi:10.1093/jac/dks121

Potron, A., Nordmann, P., Rondinaud, E., Jaureguy, F., Poirel, L., 2013. A mosaic transposon encoding OXA-48 and CTX-M-15: Towards pan-resistance. J. Antimicrob. Chemother. doi:10.1093/jac/dks397

Potron, A., Poirel, L., Bussy, F., Nordmann, P., 2011a. Occurrence of the carbapenem-hydrolyzing β-lactamase gene blaO$_{XA-48}$ in the environment in Morocco. Antimicrob. Agents Chemother. 55, 5413–5414. doi:10.1128/AAC.05120-11

Potron, A., Poirel, L., Nordmann, P., 2011b. Origin of OXA-181, an emerging carbapenem-hydrolyzing oxacillinase, as a chromosomal gene in *Shewanella xiamenensis*. Antimicrob. Agents Chemother. 55, 4405–7. doi:10.1128/AAC.00681-11

Ramírez, M.S., Merkier, A.K., Almuzara, M., Vay, C., Centrón, D., 2010. Reservoir of antimicrobial resistance determinants associated with horizontal gene transfer in clinical isolates of the genus *Shewanella*. Antimicrob. Agents Chemother. doi:10.1128/AAC.00570-10

Ribeiro, T.G., Novais, Â., Branquinho, R., Machado, E., Peixe, L., 2015. Phylogeny and comparative genomics unveil independent diversification trajectories of *qnrB* and genetic platforms within particular *Citrobacter* species. Antimicrob. Agents Chemother. 59, 5951–5958. doi:10.1128/AAC.00027-15

Romine, M.F., Carlson, T.S., Norbeck, A.D., McCue, L.A., Lipton, M.S., 2008. Identification of mobile elements and pseudogenes in the *Shewanella oneidensis* MR-1 genome. Appl. Environ. Microbiol. 74, 3257–3265. doi:10.1128/AEM.02720-07

Sampaio, J.L.M., Ribeiro, V.B., Campos, J.C., Rozales, F.P., Magagnin, C.M., Falci, D.R., Silva, R.C.F.D., Dalarosa, M.G., Luz, D.I., Vieira, F.J., Antochevis, L.C., Barth, A.L., Zavascki, A.P., 2014. Detection of OXA-370, an OXA-48-related class D β-lactamase, in *Enterobacter hormaechei* from Brazil. Antimicrob. Agents Chemother. 58, 3566–3567. doi:10.1128/AAC.02510-13

Sidjabat, H.E., Kennedy, K., Silvey, A., Collignon, P., Paterson, D.L., 2013. Emergence of $bla_{OXA-181}$-carrying ColE plasmid in *Klebsiella pneumoniae* in Australia. Int. J. Antimicrob. Agents 41, 294–296. doi:10.1016/J.IJANTIMICAG.2012.11.003

Siguier, P., Filée, J., Chandler, M., 2006. Insertion sequences in prokaryotic genomes. Curr. Opin. Microbiol. 9, 526–531. doi:10.1016/J.MIB.2006.08.005

Tacão, M., Correia, A., Henriques, I., 2013. Environmental *Shewanella xiamenensis* strains that carry bla_{OXA-48} or $bla_{OXA-204}$ genes: additional proof for bla_{OXA-48}-like gene origin. Antimicrob. Agents Chemother. 57, 6399–6400. doi:10.1128/AAC.00771-13

Tacão, M., Correia, A., Henriques, I., 2012. Resistance to broad-spectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of bla_{CTX-M}-like genes. Appl. Environ. Microbiol. 78, 4134–4140. doi:10.1128/AEM.00359-12

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Tsai, M.S., You, H.L., Tang, Y.F., Liu, J.W., 2008. *Shewanella* soft tissue infection: case report and literature review. Int. J. Infect. Dis. 12, e119–e124. doi:10.1016/j.ijid.2008.03.020

Villa, L., Carattoli, A., Nordmann, P., Carta, C., Poirel, L., 2013. Complete sequence of the IncT-type plasmid pT-OXA-181 carrying the $bla_{OXA-181}$ carbapenemase gene from *Citrobacter freundii*. Antimicrob. Agents Chemother. 57, 1965–1967. doi:10.1128/AAC.01297-12

Zhao, J.Y., Mu, X.D., Zhu, Y.Q., Xi, L., Xiao, Z., 2015. Identification of an integron containing the quinolone resistance gene *qnrA1* in *Shewanella xiamenensis*. FEMS Microbiol. Lett. 362, fnv146. doi:10.1093/femsle/fnv146

Zong, Z., 2012. Discovery of $bla_{OXA-199}$, a chromosome-based bla_{OXA-48}-like variant, in *Shewanella xiamenensis*. PLoS One 7, e48280. doi:10.1371/journal.pone.0048280

Zong, Z., 2011. Nosocomial peripancreatic infection associated with *Shewanella xiamenensis*. J. Med. Microbiol. 60, 1387–1390. doi:10.1099/jmm.0.031625-0

CHAPTER 2: *Shewanella species as progenitors of qnrA genes: insights into gene diversity*

Submitted to *Microbiology*, June 2021

1. ABSTRACT

Members of the genus *Shewanella* are ubiquitous in aquatic environments, some of which have been implicated in human infections. The progenitors of antibiotic resistance genes with clinical relevance, such as the *qnrA* genes, have been identified in *Shewanella*. *qnrA* code for a pentapeptide repeat protein that protects type II topoisomerases, decreasing bacterial susceptibility to quinolones and fluoroquinolones. In this study, 248 genomes of 49 *Shewanella* species were analyzed as well as a collection of 33 environmental isolates belonging to 10 *Shewanella* species. The presence of the *qnrA* gene was detected in 22.9% of the genomes and 15.2% of the isolates. The gene was more often detected in *Shewanella algae*, but was also detected in *Shewanella carassii*, *Shewanella chilikensis*, *Shewanella haliotis* and *Shewanella indica*. The identified genes encoded the previously described variants QnrA3 (in 22 genomes of 1 species), QnrA2 (in 8 genomes and 3 species), QnrA1 (in 6 genomes and 2 species), QnrA7 (in 5 genomes and 2 species), QnrA10 (in 2 genomes of 1 species) and QnrA4 (in 1 genome). In addition, 11 novel variants with 3 to 7 amino acid substitutions were identified (in 13 genomes and 1 environmental isolate). The presence of this gene appears to be species-specific although within each species several variants were detected. The study presents a previously unknown diversity of *qnrA* genes in *Shewanella* genomes, highlighting the role of this genus as a progenitor and reservoir of these genes. Further studies are needed to determine the phenotypes conferred by the new variants as well as the mechanisms that may mediate the transfer of these genes to new hosts.

Keywords:

Quinolone resistance; Gene progenitor; *qnrA* gene; Environmental *Shewanella*.

2. INTRODUCTION

Quinolones are among the most widely prescribed antibiotics to treat infections caused by Gram-negative and Gram-positive bacteria (Pham et al., 2019). Since their introduction more than six decades ago, they have been used because of their high potency, broad-spectrum activity, oral bioavailability, and relatively high safety profile (Janecko et al., 2016; Pham et al., 2019). Quinolones and fluoroquinolones have been extensively used in animal agriculture and aquaculture, and also in veterinary and human medicine, in which these drugs are of critical importance for multiple clinical indications (Janecko et al., 2016). Inevitably, resistance to this class of antibiotics began to spread, amongst not only clinical settings, namely in members of the *Enterobacteriaceae* family (Nordmann and Poirel, 2005; Rodríguez-Martínez et al., 2011), but also in the environment (Girijan et al., 2020).

Quinolones mode of action is based in the inhibition of the activity of type II topoisomerases, namely DNA gyrase and topoisomerase IV, encoded respectively by the *gyrA* and *gyrB* genes, and by *parC* and *parE* genes (Janecko et al., 2016). In general, these are the primary targets in Gram-negative and Gram-positive bacteria, respectively, which mutations are responsible for one of the main bacterial resistance mechanisms against quinolones. Other common quinolones chromosomally-encoded resistance mechanisms are over-expression of efflux pumps and decreased outer-membrane permeability (Poirel et al., 2008).

In addition, the emergence of several plasmid-mediated quinolone resistance (PMQR) mechanisms has been noticed, namely the expression of Qnr proteins, the aminoglycoside-modifying enzyme, AAC(6')-Ib-cr, and the efflux pumps QepA and OqxAB (Cattoir and Nordmann, 2009; Périchon et al., 2007; Yamane et al., 2007). The *qnrA*-like genes code for a 218 amino acid (aa) protein belonging to the pentapeptide family that protects type II topoisomerases from quinolone binding (Cattoir et al., 2007; Rodríguez-Martínez et al., 2011). Twelve variants of QnrA proteins are known (QnrA1 to QnrA12) (Jacoby et al., 2014). QnrA proteins complement chromosomally-encoded resistance mechanisms, increasing resistance to quinolones and fluoroquinolones (Poirel et al., 2005; Tran and Jacoby, 2002).

Since *qnrA* discovery in 1998, detected in plasmid *pMG252* from a multi-resistant strain of *Klebsiella pneumoniae* (thereafter called *qnrA1*) (Jacoby et al., 1998), *qnrA*-like genes have been found on the genomes of both Gram-positive and Gram-negative bacteria, leading to intrigue about their natural reservoir. A search for *qnrA* genes in both clinically relevant and environmental Gram-negative species (including species belonging to *Enterobacteriaceae, Aeromonadaceae, Pseudomonadaceae, Xanthomonadaeae, Moraxellaceae*, and *Shewanellaceae*) identified *S. algae* as the most probable progenitor of *qnrA* (Poirel et al., 2005). In this study we assessed the diversity of *qnrA* genes in *S. algae* and other *Shewanella* species, to better understand the role of this genus as reservoir and progenitor of these genetic determinants.

3. METHODS

3.1. *In silico* analysis of *Shewanella* spp. genomes

A total of 248 *Shewanella* genomes (60 complete, 188 draft) were retrieved from the National Centre for Biotechnology Information (NCBI) and from the Pathosystems Resource Integration (PATRIC) databases. For all *Shewanella* genomes with no previously inferred affiliation to the species level, a whole-genome based analysis against *Shewanella* type strains genomes was performed using the Type (Strain) Genome Server pipeline (Meier-Kolthoff and Göker, 2019).

All genomes were screened for the presence of *qnrA*-like genes (search conducted in October 2020). Previously described variants from clinical isolates (from *qnrA1* to *qnrA12*) (see supplemental material, Table S1) were used for the BLAST similarity search (against all *Shewanella* genomes available). Sequences showing above 90% nucleotide similarity (and high query coverage, 100%) with the *qnrA* variants were selected for further analysis.

3.2. Analysis of *qnrA*-like variants in *Shewanella* environmental isolates

A collection of environmental *Shewanella* spp. isolates (n=33) was screened

for the presence of *qnrA*-like genes. These isolates were previously retrieved from estuarine water (n=14), saltmarsh plant *Halimione portulacoides* (n=7), cockle (n=9) and river water (n= 3) (Azevedo et al., 2012; Fidalgo et al., 2016; Tacão et al., 2018). Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega), following the manufacturer's instructions. A PCR-based screening was used for *qnrA*-like genes detection in all isolates.

A previously designed set of primers (P1: 5' TTCTCACGCCAGGATTTG and P2: 5' CCATCCAGATCGGCAA; Guillard et al., 2011) was used to amplify a gene fragment with 521 bp (Supplemental material, Figure S1). PCR reaction mixtures had the following composition: NZYTaq 2X Green Master Mix (6.25 µl; 2.5 mM $MgCl_2$, 200 µM dNTPs, 1.25 U polymerase; NZYTech), 0.75 µL of each primer (10 mM), 1 µl of purified DNA (50–100 ng), and nuclease-free water up to a final volume of 25 µl. The temperature profile was as described previously by Guillard et al., 2011. For the isolates for which a positive result was obtained, the complete gene was amplified with primers designed in this study (P3: 5' CCGATGTTAGCCTGCAAAG and P4: 5' CCTTTGAAGGGAAGGTATAAC) targeting regions upstream and downstream the gene (Figure S1). PCR reaction mixtures (25 µl) were as described above. PCR program was as follows: initial denaturation (94°C for 9 min); 30 cycles of denaturation (94°C for 30 s), annealing (50°C for 30 s), and extension (72°C for 1 min); and a final extension (72°C for 10 min); The resulting amplicon with ~1052 bp was sequenced.

3.3. Phylogenetic analysis

Deduced amino acid sequences were aligned by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic analysis was based on QnrA-like representative variants retrieved from GenBank database, new variants identified *in silico*, and variants obtained from the *Shewanella* environmental isolates. The phylogenetic tree was built using software MEGA version 6 (Tamura et al., 2013) based in multiple protein sequence alignment with the maximum likelihood method using 1000 bootstrap replicates.

4. RESULTS AND DISCUSSION

The *Shewanella* genus includes at least 70 validly published species (Lemaire et al., 2020). *Shewanella* members are known to be ubiquitous, typically found inhabiting all sorts of aquatic environments, from the deep sea, to intertidal zones, estuaries and sediments (Gao et al., 2006; Lemaire et al., 2020). Some species have the ability to cause infections in humans, namely *S. algae* (Janda, 2014; Tsai et al., 2008; Tseng et al., 2018), *Shewanella putrefaciens* (Janda, 2014; Tsai et al., 2008), *Shewanella xiamenensis* (Antonelli et al., 2015; Zong, 2011), *Shewanella haliotis* (Poovorawan et al., 2013) and *Shewanella oneidensis* (Venkateswaran et al., 1999). This study analyzed the diversity of *qnrA*-like genes in 248 genomes, including 166 genomes belonging to 49 species, and 82 genomes whose species-affiliation was not previously determined. Additionally, 33 previously identified environmental *Shewanella* isolates (see supplemental material, Table S1), belonging to 10 species, were inspected for the presence and diversity of *qnrA*-like genes.

The genome-based screening enabled the detection of a *qnrA*-like gene in 57 genomes (22.9% of the total; Table 1). Of these, 7 were detected in unassigned *Shewanella* genomes. Thus, the TYGS whole genome analysis was performed and corresponding strains were affiliated to *S. algae* (n=3), *S. indica* (n=3) or *S. chilikensis* (n=1). Hence, a *qnrA*-like gene was detected in *S. algae* (in 45 out of 46 *S. algae* genomes), *S. carassii* (in 2 out of 2), *S. chilikensis* (in 4 out of 4), *S. haliotis* (in 1 out of 1) and *S. indica* (in 5 out of 5) (Table 1). Genes detected in 39% of the *qnrA*-positive genomes were closely related (above 99% similarity in terms of nucleotide sequence) to *qnrA3*. This gene variant was detected among *S. algae* genomes (corresponding to 49% of all *S. algae* genomes analyzed). The remaining genes detected were closely related with *qnrA2* (in 14% of the *qnrA*-positive genomes), most of which were identified as *S. indica*, but also *S. algae* and *S. chilikensis*; *qnrA1* was detected in *S. algae* and *S. chilikensis*; *qnrA10* was detected in *S. algae*; *qnrA7* detected in *S. algae* and *S. haliotis*; and *qnrA4* detected in *S. algae*. In terms of the deduced amino acid sequence, sequences identical to 6 of the 12 variants previously reported were detected, namely QnrA1 (encoded in 3 genomes of *S. chilikensis* and 3 genomes of *S. algae*), QnrA2 (in all 5 genomes of *S. indica*, 2 of *S. algae* and 1 in *S. chilikensis*), QnrA3 (encoded in

22 genomes of *S. algae*), QnrA4 (1 *S. algae*), QnrA7 (4 *S. algae* and 1 *S. haliotis*) and QnrA10 (2 *S. algae*). In addition, 10 new variants were detected (Table 1; Figure 1). All the new deduced protein variants identified presented the typical pentapeptide repeating units of the Qnr proteins (Figure 1), with consensus sequence of S/T/A/V/C-D/N-L/F-S/T/R-G, in two domains separated by a single amino acid (glycine) (Strahilevitz et al., 2009). New variants presented from 3 up to 6 aa substitutions when compared to QnrA1 (Figure 1), and were located in 11 pentapeptide motifs in which amino acid substitutions were never described.

TABLE 1. *qnrA*-like variants detected in *Shewanella* genomes currently available in public databases and number of amino acids substitutions in the translated sequence.

Accession number	Affiliation	Strain	Genome status	QnrA	aa substitutions in new variant[a]
BAXN01000002	*S. algae*	JCM 19057	Draft	nv1	5 (Q39R, V108I, T127A, N173D, G176S)
CP047422	*S. algae*	18064-CSB-B-B	Complete	QnrA3	-
NIJL01000056	*S. algae*	20-23R	Draft	nv2	4 (Q39R, S60N, V108I, T127A)
CP055159	S. algae	2NE11	Complete	nv3	6 (R27C, Q39R, S42C, V108I, T127A, G131S)
JADP00000000	*S. algae*	38A_GOM-205m	Draft	QnrA3	-
JACDTT010000001	*S. algae*	A3/19	Complete	QnrA7	-
LVDH01000001	*S. algae*	AC	Draft	QnrA3	-
LVCY01000001	*S. algae*	ACCC	Draft	QnrA1	-
JAAXPX010000001	*S. algae*	ATCC 51192	Draft	QnrA3	-
MDKA01000048	*S. algae*	BrY	Draft	QnrA2	-
JPMA01000007	*S. algae*	C6G3	Draft	nv4	4 (Q39R, V108I, T127A, G162S)
CP018456	*S. algae*	CCU101	Complete	QnrA7	-
CP068230	*S. algae*	CECT 5071	Complete	QnrA3	-
LVDF01000001	*S. algae*	CHL	Draft	QnrA3	-
LTBI01000001	*S. algae*	CLS1	Draft	nv5	4 (Q39R, A97V, V108I, T127A)
LVDV01000001	*S. algae*	CLS2	Draft	QnrA3	-
LVDX01000001	*S. algae*	CLS3	Draft	QnrA3	-
LVDD01000001	*S. algae*	CLS4	Draft	QnrA3	-
LVDE01000001	*S. algae*	CLS5	Draft	QnrA7	-
MBFW01000016	*S. algae*	CSB04KR	Draft	QnrA2	-
JAAUHW010000001	*S. algae*	Iso12	Draft	QnrA3	-
BALO01000046	*S. algae*	JCM 21037	Draft	QnrA3	-
LUJI01000001	*S. algae*	JFC1	Draft	QnrA3	-
LUKM01000001	*S. algae*	JFC2	Draft	QnrA3	-
LVCX01000001	*S. algae*	JFC3	Draft	QnrA3	-
LVDI01000001	*S. algae*	JFL	Draft	nv6	5 (Q39R, A97T, V108I, T127A, S145N)

CP033575	*S. algae*	KC-Na-R1	Complete	nv7	6 (Q39R, A67T, A82T, V108I, T127A, V213I)
CDQH01000000	*S. algae*	MARS 14	Draft	QnrA10	-
LVDG01000001	*S. algae*	melkephyllucas	Draft	nv8	4 (Q39R, V54I, V108I, T127A)
LIRM01000001	*S. algae*	MN-01	Draft	QnrA3	-
BCZT01000049	*S. algae*	NBRC 103173	Draft	QnrA3	-
UGYO01000001	*S. algae*	NCTC10738	Draft	QnrA3	-
LVCZ01000001	*S. algae*	RC	Draft	QnrA3	-
CP046378	*S. algae*	RQs-106	Complete	nv9	5 (Q39R, S42C, V108I, T127A, G131S)
QFDC01000001	*S. algae*	Sh392	Draft	QnrA7	-
LVDC01000001	*S. algae*	SYC	Draft	QnrA1	-
LUCP01000001	*S. algae*	SYT1	Draft	QnrA3	-
LVDT01000001	*S. algae*	SYT2	Draft	QnrA4	-
LVDW01000001	*S. algae*	SYT3	Draft	nv8	4 (Q39R, V54I, V108I, T127A)
LVDK01000001	*S. algae*	SYT4	Draft	nv1	5 (Q39R, V108I, T127A, N173D, G176S)
LVDS01000001	*S. algae*	TYL	Draft	QnrA3	-
CP034246	*S. algae*	VGH117	Complete	QnrA10	-
LVDU01000001	*S. algae*	YHL	Draft	QnrA3	-
LVDA01000001	*S. algae*	YTH	Draft	QnrA3	-
LVDB01000001	*S. algae*	YTL	Draft	QnrA1	-
NGVS01000001	*S. carassii*	08MAS2251	Draft	nv10	3 (Q39R, S81N, T127A)
BMKO01000006	*S. carassii*	CGMCC	Draft	nv10	3 (Q39R, S81N, T127A)
CP045857	*S. chilikensis*	DC57	Complete	QnrA2	-
NIJM01000001	*S. chilikensis*	JC5	Draft	QnrA1	-
BMXX01000043	*S. chilikensis*	KCTC 22540	Draft	QnrA1	-
DPAE01000001	*S. chilikensis*	UBA12176	Draft	QnrA1	-
BALL01000013	*S. haliotis*	JCM 14758	Draft	QnrA7	-
JWGX01000033	*S. indica*	ECSMB14102	Draft	QnrA2	-
BMYE01000001	*S. indica*	KCTC 23171	Draft	QnrA2	-
NIJK01000001	*S. indica*	KJW27	Draft	QnrA2	-
LVDR01000001	*S. indica*	MSW	Draft	QnrA2	-
JAAEJW010000001	*S. indica*	SE1	Draft	QnrA2	-

[a]aa substitutions relative to variant QnrA1

From the collection of 33 *Shewanella* isolates, *qnrA* was detected in 5 isolates (Table 1). These isolates affiliated to three *Shewanella* species: *S. algae* (isolate Sh2), *S. haliotis* (isolates Sh4 and Sh14) and *S. indica* (isolates Sh15 and Sh16). Sequence analysis revealed that genes encoded enzymes 100% identical to QnrA2 (Sh15 and Sh16), QnrA3 (Sh14) and QnrA12 (Sh2). In isolate *S. haliotis* Sh4 a new variant was detected (hereafter designated nv11; Figure 1), with 98% nucleotide similarity with the gene *qnrA3*. In terms of the deduced amino acid sequence, when compared to QnrA1 the encoded protein presented 7 aa substitutions (L18V, F23L, Q39R, G52S, F79L, V108I, T127A), 4 of which were never detected before (L18V, F23L, G52S and F79L), and 3 of which were located in pentapeptide motifs in which amino acid substitutions have never been identified.

A phylogenetic tree was built based on the deduced amino acid sequences of all the *qnrA* sequences detected in the genomes analyzed, as well as those detected in the environmental isolates (Figure 2). In this tree, the representative sequences of the variants described so far (QnrA1 to QnrA12), obtained from the GenBank database, were also included. It is patent the dominance of the variant QnrA3 in the *Shewanella* genomes. The frequent occurrence of these sequences in *S. algae* genomes is also evidenced. *S. algae* was recently highlighted as the causing agent of the vast majority of infections attributed to *Shewanella* isolates (Janda, 2014; Tseng et al., 2018), contrary to previous estimates that pointed to *S. putrefaciens* as the most common pathogen within this genus (Janda, 2014). *S. algae* infections are usually opportunistic and waterborne (Tseng et al., 2018), and include skin and soft tissue infections as well as bacteremia (Tsai et al., 2008). From the remaining species here identified as carriers of the *qnrA* gene, *S. haliotis* has also been associated to human opportunistic infections (Poovorawan et al., 2013).

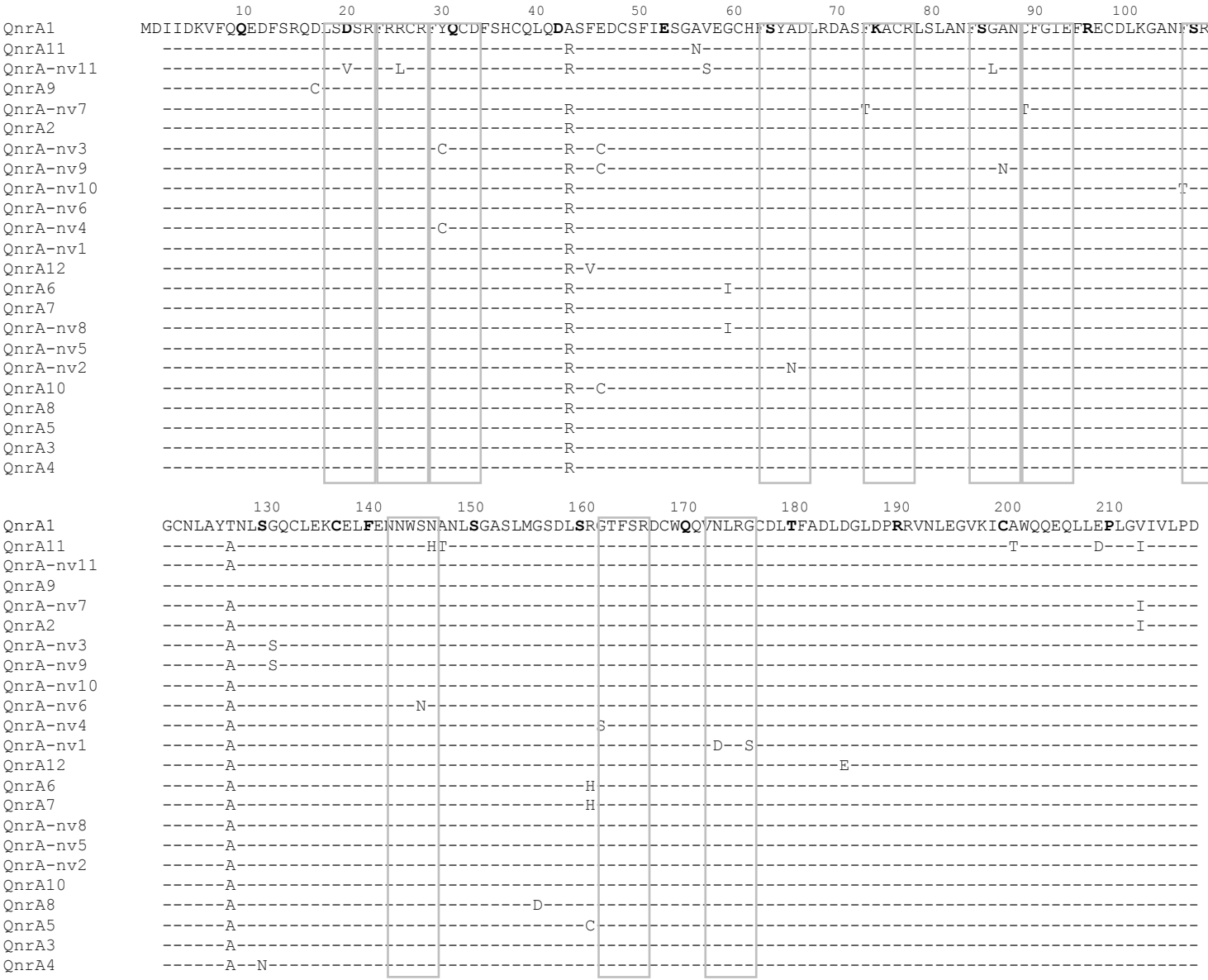


FIGURE 1. Deduced amino acid sequences alignment of new QnrA-like determinants identified in *Shewanella* genomes (QnrA-nv1 to nv-10), in *S. haliotis* Sh4 isolate (QnrA-nv11) and known QnrA variants (QnrA1 to QnrA12, accession numbers: AAL60061, AAT79355, AAZ04782, AAZ04783, AAZ04784, AAZ78355, ACV83303, ATU31384, PIA06137, AZP56653, QMU53430, QMU53432). Dashes are for identical amino acid residues. Pentapeptide motifs in which new amino acid

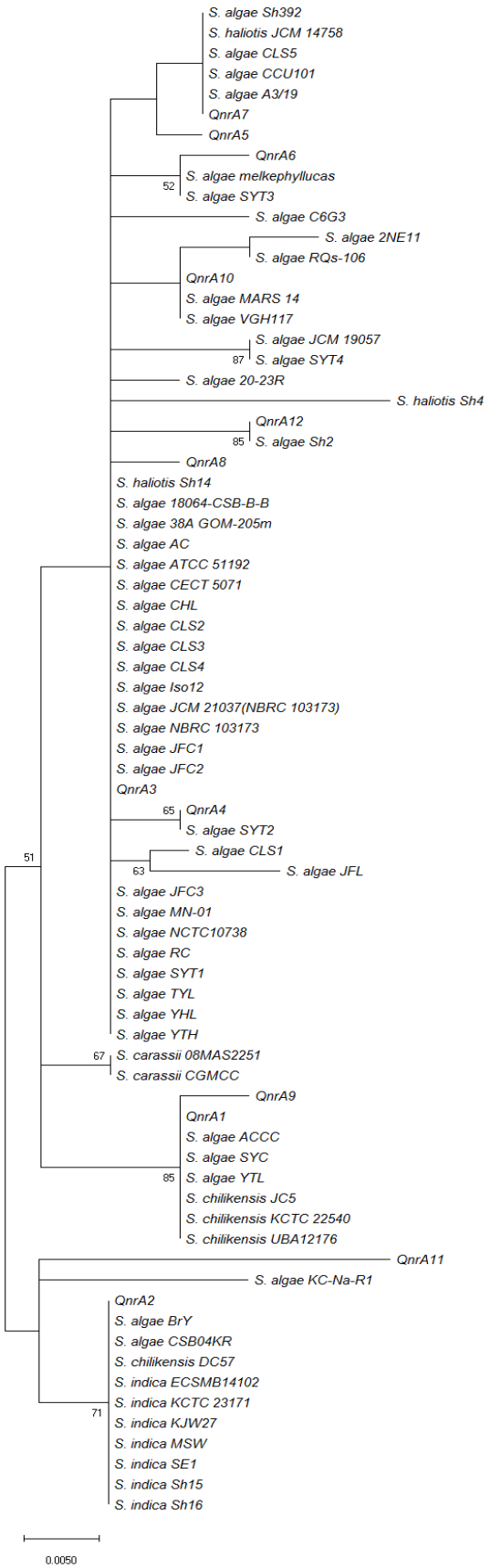


Figure 2. Phylogenetic tree based on the QnrA deduced amino acid sequences identified in *Shewanell*a in this study, together with previously described QnrA variants. The tree was built with the maximum likelihood method with 1000 bootstrap replicas.

5. CONCLUSION

Overall, the results presented in this study reinforce the role of *Shewanella* genus as the progenitor of *qnrA* genes. However, its presence seems to be species-related, intrinsic to some *Shewanella* species. From the 49 species represented in our dataset, the gene was not detected in 44 species. Among the species here identified as *qnrA* carriers, this gene has been previously reported in the genome of *S. algae* (Melvold et al., 2017; Poirel et al., 2005), *S. xiamenensis* (Zhao et al., 2015), but to our knowledge never in *S. carassii*, *S. chilikensis* and *S. haliotis* and *S. indica*. This study also revealed a great and unknown diversity of *qnrA*-like genes in strains of the genus *Shewanella*, demonstrating its relevance as reservoir of genes that may eventually reach clinical settings and become a potential danger to human health. Variants were in general not species-specific. For instance, in *S. algae* 6 distinct variants were detected but, in turn, in *S. indica* *qnrA2* gene was the only variant detected.

The resistance phenotype associated with these new variants as well as the mechanisms underlying their transfer to other bacteria should be investigated in the future in order to anticipate possible human health risks.

6. ACKNOWLEDGMENTS

The authors acknowledge the financial support provided by FCT (Fundação para a Ciência e a Tecnologia) through CESAM funds (UIDP/50017/2020+ UIDB/50017/2020) and individual grants to Marta Tacão (CEECIND/00977/2020) and Susana Araújo (SFRH/BD/52573/2014).

7. REFERENCES

Antonelli, A., Di Palo, D.M., Galano, A., Becciani, S., Montagnani, C., Pecile, P., Galli, L., Rossolini, G.M., 2015. Intestinal carriage of *Shewanella xiamenensis* simulating carriage of OXA-48–producing *Enterobacteriaceae*. Diagn. Microbiol. Infect. Dis. 82, 1–3. doi:10.1016/j.diagmicrobio.2015.02.008

Azevedo, J.S.N., Ramos, I., Araújo, S., Oliveira, C.S., Correia, A., Henriques, I.S., 2012. Spatial and temporal analysis of estuarine bacterioneuston and bacterioplankton using culture-dependent and culture-independent methodologies. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 101, 819–835. doi:10.1007/s10482-012-9697-z

Cattoir, V., Nordmann, P., 2009. Plasmid-mediated quinolone Resistance in Gram-negative bacterial species: An Update. Curr. Med. Chem. 16, 1028–1046. doi:10.2174/092986709787581879

Cattoir, V., Poirel, L., Rotimi, V., Soussy, C.J., Nordmann, P., 2007. Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates. J. Antimicrob. Chemother. 60, 394–397. doi:10.1093/jac/dkm204

Fidalgo, C., Henriques, I., Rocha, J., Tacão, M., Alves, A., 2016. Culturable endophytic bacteria from the salt marsh plant *Halimione portulacoides*: phylogenetic diversity, functional characterization, and influence of metal(loid) contamination. Environ. Sci. Pollut. Res. 23, 10200–10214. doi:10.1007/s11356-016-6208-1

Gao, H., Obraztova, A., Stewart, N., Popa, R., Fredrickson, J.K., Tiedje, J.M., Nealson, K.H., Zhou, J., 2006. *Shewanella loihica* sp. nov., isolated from iron-rich microbial mats in the Pacific Ocean. Int. J. Syst. Evol. Microbiol. 56, 1911–1916. doi:10.1099/ijs.0.64354-0

Girijan, S.K., Paul, R., Rejish Kumar, V.J., Pillai, D., 2020. Investigating the impact of hospital antibiotic usage on aquatic environment and aquaculture systems: A molecular study of quinolone resistance in *Escherichia coli*. Sci. Total Environ. 748, 141538. doi:10.1016/j.scitotenv.2020.141538

Guillard, T., Moret, H., Brasme, L., Carlier, A., Vernet-Garnier, V., Cambau, E., de Champs, C., 2011. Rapid detection of *qnr* and *qepA* plasmid-mediated quinolone resistance genes using real-time PCR. Diagn. Microbiol. Infect. Dis. 70, 253–259. doi:10.1016/j.diagmicrobio.2011.01.004

Jacoby, G.A., Martinez-Martinez, L., Pascual, A., 1998. Quinolone resistance from a transferable plasmid. Lancet 351, 797–799.

Jacoby, G.A., Strahilevitz, J., Hooper, D.C., 2014. Plasmid-mediated quinolone resistance. Microbiol. Spectr. 2, 1–42. doi:10.1128/microbiolspec.PLAS-0006-2013

Janda, J.M., 2014. *Shewanella*: A marine pathogen as an emerging cause of human disease. Clin. Microbiol. Newsl. 36, 25–29. doi:10.1016/j.clinmicnews.2014.01.006

Janecko, N., Pokludova, L., Blahova, J., Svobodova, Z., Literak, I., 2016. Implications of fluoroquinolone contamination for the aquatic environment—A review. Environ. Toxicol. Chem. doi:10.1002/etc.3552

Lemaire, O.N., Méjean, V., Iobbi-Nivol, C., 2020. The *Shewanella* genus: ubiquitous organisms sustaining and preserving aquatic ecosystems. FEMS Microbiol. Rev. 44, 155–170. doi:https://doi.org/10.1093/femsre/fuz031

Meier-Kolthoff, J.P., Göker, M., 2019. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. Nat. Commun. 10, 2182. doi:10.1038/s41467-019-10210-3

Melvold, J.A., Wyrsch, E.R., McKinnon, J., Chowdhury, P.R., Charles, I.G., Djordjevic, S.P., 2017. Identification of a novel *qnrA* allele, *qnrA8*, in environmental *Shewanella algae*. J. Antimicrob. Chemother. 72, 2949–2952. doi:10.1093/jac/dkx226

Nordmann, P., Poirel, L., 2005. Emergence of plasmid-mediated resistance to quinolones in *Enterobacteriaceae*. J. Antimicrob. Chemother. doi:10.1093/jac/dki245

Périchon, B., Courvalin, P., Galimand, M., 2007. Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in *Escherichia coli*. Antimicrob. Agents Chemother. 51, 2464–2469. doi:10.1128/AAC.00143-07

Pham, T.D.M., Ziora, Z.M., Blaskovich, M.A.T., 2019. Quinolone antibiotics. Medchemcomm 10, 1719–1739. doi:10.1039/c9md00120d

Poirel, L., Cattoir, V., Nordmann, P., 2008. Is plasmid-mediated quinolone resistance a clinically significant problem? Clin. Microbiol. Infect. doi:10.1111/j.1469-0691.2007.01930.x

Poirel, L., Liard, A., Nordmann, P., Mammeri, H., 2005. Origin of plasmid-mediated quinolone resistance determinant QnrA. Antimicrob. Agents Chemother. 49, 3523–3525. doi:10.1128/AAC.49.8.3523

Poovorawan, K., Chatsuwan, T., Lakananurak, N., Chansaenroj, J., Komolmit, P., Poovorawan, Y., 2013. *Shewanella haliotis* associated with severe soft tissue infection, Thailand, 2012. Emerg. Infect. Dis. doi:10.3201/eid1906.121607

Rodríguez-Martínez, J.M., Cano, M.E., Velasco, C., Martínez-Martínez, L., Pascual, Á., 2011. Plasmid-mediated quinolone resistance: An update. J. Infect. Chemother. 17, 149–182. doi:10.1007/s10156-010-0120-2

Strahilevitz, J., Jacoby, G.A., Hooper, D.C., Robicsek, A., 2009. Plasmid-mediated quinolone resistance: A multifaceted threat. Clin. Microbiol. Rev. doi:10.1128/CMR.00016-09

Tacão, M., Araújo, S., Vendas, M., Alves, A., Henriques, I., 2018. *Shewanella* species as the origin of $bla_{\text{OXA-48}}$ genes: insights into gene diversity, associated phenotypes and possible transfer mechanisms. Int. J. Antimicrob. Agents 51, 340–348. doi:10.1016/j.ijantimicag.2017.05.014

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729. doi:10.1093/molbev/mst197

Tran, J.H., Jacoby, G.A., 2002. Mechanism of plasmid-mediated quinolone resistance. Proc. Natl. Acad. Sci. U. S. A. 99, 5638–5642. doi:10.1073/pnas.082092899

Tsai, M.S., You, H.L., Tang, Y.F., Liu, J.W., 2008. *Shewanella* soft tissue infection: case report and literature review. Int. J. Infect. Dis. 12, e119–e124. doi:10.1016/j.ijid.2008.03.020

Tseng, S.Y., Liu, P.Y., Lee, Y.H., Wu, Z.Y., Huang, C.C., Cheng, C.C., Tung, K.C., 2018. The pathogenicity of *Shewanella algae* and ability to tolerate a wide range of temperatures and salinities. Can. J. Infect. Dis. Med. Microbiol. 2018, 6976897. doi:10.1155/2018/6976897

Venkateswaran, K., Moser, D.P., Dollhopf, M.E., Lies, D.P., Saffarini, D.A., MacGregor, B.J., Ringelberg, D.B., White, D.C., Nishijima, M., Sano, H., Burghardt, J., Stackebrandt, E., Nealson, K.H., 1999. Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. Int. J. Syst. Bacteriol. 49, 705–724. doi:10.1099/00207713-49-2-705

Yamane, K., Wachino, J.I., Suzuki, S., Kimura, K., Shibata, N., Kato, H., Shibayama, K., Konda, T., Arakawa, Y., 2007. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. Antimicrob. Agents Chemother. 51, 3354–3360. doi:10.1128/AAC.00339-07

Zhao, J.Y., Mu, X.D., Zhu, Y.Q., Xi, L., Xiao, Z., 2015. Identification of an integron containing the quinolone resistance gene qnrA1 in *Shewanella xiamenensis*. FEMS Microbiol. Lett. 362, fnv146. doi:10.1093/femsle/fnv146

Zong, Z., 2011. Nosocomial peripancreatic infection associated with *Shewanella xiamenensis*. J. Med. Microbiol. 60, 1387–1390. doi:10.1099/jmm.0.031625-0

II.2. IMPACT OF ANTHROPOGENIC ACTIVITIES IN THE DISSEMINATION OF ANTIBIOTIC RESISTANCE

(CHAPTER 3, CHAPTER 4 and CHAPTER 5)

CHAPTER 3: *Characterization of antibiotic resistant and pathogenic Escherichia coli in irrigation water and vegetables in household farms*

International Journal of Food Microbiology, 2017
doi: 10.1016/j.ijfoodmicro.2017.06.020

1. ABSTRACT

This study aimed to characterize *Escherichia coli* present in irrigation water and vegetables from 16 household farms. Isolates were obtained from 50% of water (n=210 isolates) and 38% of vegetable samples (n=239). Phylogroups B1 (56% of isolates) and A (22%) were the most prevalent both in water and vegetables. Diarrheagenic strains were detected in vegetables. Irrespective of the source (i.e. water or vegetables), the most common antibiotic resistance was against streptomycin (89% resistant isolates) and tetracycline (24%). Common acquired genes (e.g. bla_{TEM}, *tetA*, *tetB*) were found in isolates from both sources. Class I integrons were detected in water (arrays *dfrA1-aadA1* and *dfr16-blaP1b-aadA2-ereA*) and vegetables (unknown arrays). *intI2* was detected in water (*dfrA1-sat2-aadA1*). Plasmids were detected in 14 isolates (IncFIC, IncFIB, IncFrep, IncI1 in both samples; IncY in vegetables). Plasmids from seven isolates were transferrable by conjugation, conferring resistance to antibiotics to the recipient strain. Multidrug-resistant (MDR) strains were isolated from water (12% of the unique isolates) and vegetables (21%). Predominant sequence types (STs) among MDR isolates were ST10, ST297 and ST2522. In some cases, the same STs and identical clones (as showed by rep-PCR typing) were detected in water and vegetables, suggesting cross-contamination. This study identified several risk factors in *E. coli* isolates from vegetables and irrigation water, raising health concerns. Also, results suggest that irrigation groundwater constitutes a source of *E. coli* that may enter the food chain through vegetables ingestion.

Keywords:

Aquatic compartments; Antibiotic resistance; Vegetables contamination; Irrigation water; *Escherichia coli*.

2. INTRODUCTION

In the past decades an extra effort in promoting balanced diets has been made by health authorities worldwide. Fresh produce, being a good source of essential components (i.e. vitamins, minerals and phytonutrients), protect against a range of illnesses such as cancers, cardiovascular diseases, diabetes and obesity (Pomerleau et al., 2006). In Europe, the Food and Agriculture Organization (FAO) indicates that fresh produce consumption has increased over the last four decades, and that in Southern Europe countries, including Portugal, the consumption of vegetables is higher than in Northern Europe (Elmadfa et al., 2009).

Fresh produce naturally carry a non-pathogenic microbial community but may become contaminated with human pathogens (Boehme et al., 2004; Edelstein et al., 2014). Contaminated fresh produce, and in particular leafy greens usually consumed raw or minimally processed, represent a risk of infection for consumers (FAO/WHO, 2008). Contamination can occur both at pre-harvest (i.e. through manure fertilization, irrigation water and wild animals) (Beuchat and Ryu, 1997) and post-harvest (i.e. by washing, handling and processing food) (Berger et al., 2010; De Roever, 1999). Despite the fact that tight food safety regulations were implemented in most countries, produce-associated outbreaks have been increasing in recent years (Jung et al., 2014). Factors contributing to the increase of produce-associated outbreaks include the production of vegetables in household farms, mainly for self-consumption, in which microbiological quality is rarely monitored.

Water used for irrigation has been identified as a main contributor to the contamination of fresh produce [Biological Hazards (BIOHAZ) Panel et al., 2013; De Roever, 1999]. Irrigation water may become contaminated either through direct contact with sewage and manure, or through nonpoint pollution sources, such as agricultural run-offs (Beuchat and Ryu, 1997; De Roever, 1999). Because water management and monitoring were inadequate in many member states, the European Union Water Framework Directive, adopted in 2000, established a framework of water policy (European Community, 2000). Groundwater use for irrigation in agriculture was stated as one important "hidden" resource. However, groundwater may also be impacted by anthropogenic activities and this impact

may persist for a long period, even for several years after the eradication of the pollution source (European Commission, 2008).

Besides contributing to the spread of pathogens, irrigation water may play a key role in the dissemination of antibiotic resistance (De Roever, 1999; LeJeune et al., 2001). The selective pressure of contaminants in aquatic systems can potentiate and accelerate the transfer of genetic resistance determinants between antibiotic-resistant bacteria (ARB) and indigenous bacteria (Lupo et al., 2012; Tacão et al., 2012). The transfer of ARB and antibiotic resistance genes (ARGs) from the environment to humans represents a great concern and may occur for example through the consumption of contaminated fresh produce.

A wide spectrum of microorganisms including bacteria, viruses and protozoa have been associated with foodborne-outbreaks (De Roever, 1999). *Enterobacteriaceae* members are the most common bacterial agents causing food poisoning outbreaks, associated with the consumption of fresh and minimally processed vegetables (Beuchat, 2002; Friesema et al., 2008; Hamilton-Miller and Shah, 2001; Hilborn et al., 1999; Söderström et al., 2008). *Escherichia coli* is a key organism in foodborne illnesses (National Center for Emerging and Zoonotic Infectious Diseases, 2011) and some strains have been implicated in international scale outbreaks (Beuchat, 1996). It is also a common indicator organism of fecal contamination in aquatic systems, and is recognized as an important player in the spread of antibiotic resistance (Henriques et al., 2006; Szmolka and Nagy, 2013). The plasticity of this species is mainly due to a high aptitude to acquire genetic information through horizontal gene transfer. Pathogenic *E. coli*, particularly those that cause foodborne illness by disrupting the normal function of the intestines, as diarrheagenic *E. coli* (DEC) strains, possess virulence traits allowing their attachment to the human gut (Kaper et al., 2004; Nataro and Kaper, 1998). Multiple outbreaks of DEC infections linked to consumption of leafy green vegetables have been reported (Edelstein et al., 2014; Friesema et al., 2008; Hilborn et al., 1999; Söderström et al., 2008).

In Portugal, legislation for monitoring irrigation water quality includes physical (e.g. pH and salinity), chemical (e.g. Cl, SO_4, Mn) and biological (e.g. fecal coliforms) parameters (Ministério do Ambiente, 1998). Parameters related to antibiotic resistance or strain virulence are not included. In addition, domestic

production of fresh produce is rarely (if ever) monitored. In Portugal, about 40% of the total population lives in rural areas, where domestic agriculture is of crucial significance (Direção-Geral De Agricultura, 2003). In this study, we characterized *E. coli* from irrigation water and vegetables from household producers, in terms of their antibiotic resistance phenotypes and genotypes, virulence determinants and the presence and diversity of mobile genetic elements. Results were analyzed in order to evaluate if irrigation groundwater represents a route of contamination of fresh produce.

3. METHODS

3.1. Study area, sampling and water quality assessment

Sampling sites were located in Estarreja, a city in the North of Portugal (Figure S1). Samples were picked from 16 household farms from June to September 2014. From each farm we sampled fresh vegetables and irrigation water used to irrigate those vegetables, from either shallow or deep wells with location never exceeding 50 meters away from the vegetables cultivation site. Vegetables collected in each farm varied from collard, cucumber, lettuce, tomato and spinach, depending on their availability at the time of sampling. All samples were collected in sterile containers, stored under refrigeration, and processed within 24h. Vegetable samples were processed following safe handling procedures recommended for human consumption purposes (e.g. handled after hand washing, trimmed of spoiled parts and washed thoroughly under running water).

Water quality was assessed through physical, chemical and microbiological parameters recommended by the Portuguese law (Ministério do Ambiente, 1998), as well as through the determination of additional parameters (e.g. NO_4, NO_2, K, Mg, Si, Ag, total coliforms and fecal enterococci).

3.2. *Escherichia coli* isolation

Five grams of each vegetable were aseptically weighed and washed with 40 mL of phosphate-buffered saline (PBS), at low speed for 10 min in a laboratory platform rocker. The volumes of 1 and 30 mL of the washing solution were filtered through 0.45 µm nitrocellulose membrane filters (Pall Corporation, USA). Water volumes of 100 and 500 mL were filtered through 0.45 µm membrane filters (Pall Corporation, USA). The filters were placed on HiCrome *E. coli* agar B (HEA) (Sigma-Aldrich, USA) plates and incubated at 44°C during 18 to 24h. After colony counting, characteristic colonies colored blue were selected and purified on HEA and Chromocult Coliform Agar (Merck, Germany) and stored in 15% glycerol at -80 °C.

3.3. Genomic fingerprinting by rep-PCR

Whole-cell suspensions were prepared in 20 µL of sterile deionized water and 1 µL of each suspension was used as DNA template for BOX-PCR fingerprinting analysis. The PCR reaction and conditions were as previously described (Araújo et al., 2014). In each PCR assay, one positive control strain was added. Band patterns were analyzed using GelCompar II version 6.1 (Applied Maths, Belgium). The similarity between profiles was calculated with the Pearson coefficient and cluster analysis was performed using the unweighted pair group method using arithmetic averages (UPGMA). Isolates displaying different BOX-PCR profiles were considered non-clonal and used for further characterization. On the other hand, isolates displaying similar BOX-PCR profiles were subjected to further analysis with ERIC- and REP-PCR fingerprinting (Araújo et al., 2014) to confirm clonality.

3.4. Determination of *E. coli* phylogenetic groups

The quadruplex PCR assay developed and revised by Clermont et al. with primers for genes *arpA, chuA, yjaA*, and for the DNA fragment TspE4.C2 was performed, to assign each isolate to one of the eight *E. coli* phylogroups previously recognized (Clermont et al., 2013). For each PCR reaction, 3 µL of cell suspensions (prepared in 20 µL of sterile deionized water) were used as template. All PCR reactions were carried out in a 25 µL volume containing NZY*Taq* 2×

Green Master Mix (2.5 mM $MgCl_2$; 200 µM dNTPs; 0.2 U/µL DNA polymerase) (NZYtech, Portugal). Primers concentration and PCR conditions were as previously described (Clermont et al., 2013). Positive and negative controls were included in each assay.

3.5. Antibiotic susceptibility testing

Escherichia coli isolates were tested for susceptibility against 16 antibiotics by the disk diffusion method on Mueller-Hinton Agar (Oxoid, Basingstoke, UK) according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015). The following antibiotics were used: amoxicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), piperacillin (30 µg), piperacillin/tazobactam (30/6 µg), cefepime (30 µg), ceftazidime (10 µg), cefotaxime (5 µg), imipenem (10 µg), aztreonam (30 µg), gentamicin (10 µg), streptomycin (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), tetracycline (30 µg), chloramphenicol (30 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg) (Oxoid, UK). *Escherichia coli* ATCC 25922 was used as quality control. Isolates were classified as sensitive or resistant according to the EUCAST recommendations after 18-24h incubation at 37°C. Growth rank between sensitive and resistant values was considered as intermediate resistance and for calculation purposes as non-susceptible. Clinical and Laboratory Standards Institute (CLSI) recommendations were used when antibiotic breakpoints in EUCAST guidelines were absent (i.e. for streptomycin, nalidixic acid, and tetracycline).

3.6. Antibiotic resistance genes and integrons detection

Escherichia coli strains displaying resistance or intermediate profiles were screened by PCR for the detection of genes conferring resistance to: β-lactams (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{GES}$, *bla*$_{AmpC}$-like), tetracycline [*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(M)], quinolones (*qnrA*, *qnrB* and *qnrS* genes and *gyrA and parC* mutations), sulfonamides (*sul1*, *sul2, sul3, dfrA1*) and aminoglycosides (a*adA1, aadA2, aadB, strA/B, aac-cr*). Primers and PCR conditions are presented in Table S2. Negative and positive controls were included in each PCR experiment. Results were confirmed by electrophoresis and

sequencing. PCR products were purified with DNA Clean & Concentrator kit (Zymo Research, USA) following manufacturer's instructions, and used as template in the sequencing reactions. Sequence similarity searches were performed against the GenBank database using BLAST software (Altschul et al., 1997). The presence of integrons was assessed through PCR amplification of *intI1*, *intI2* and *intI3* integrase genes (Table S2). The variable regions of integrase-positive strains were amplified by PCR using Extensor Long PCR Master Mix (ABgene, UK) and several combinations of primers (Table S2), and further sequenced.

3.7. Plasmid analysis

Plasmid DNA was purified using the E.Z.N.A. Plasmid Mini Kit II (Omega Bio-Tek, USA), according to the instructions, and visualized by electrophoresis in agarose gels.

Plasmid positive isolates were inspected by PCR as described previously (Carattoli et al., 2005; Moura et al., 2012b) for the detection of replicons of the following incompatibility groups: IncA/C, IncB/O, IncF (FIA, FIB, FIC, FIIA, FrepB subgroups), IncHI1, IncHI2, IncI1-Ig, IncK, IncL/M, IncN, IncP IncT, IncW and IncY.

3.8. Conjugation assays

Plasmid positive isolates were used as donors in mating assays using the rifampicin- and kanamycin-resistant strain *E. coli* CV601, according to previously described procedures (Moura et al., 2012a). Transconjugants were selected on Luria-Bertani agar plates supplemented with rifampicin (100 µg/mL), kanamycin (100 µg/mL) and tetracycline (60 µg/mL) or streptomycin (50 µg/mL). Putative transconjugants were verified by BOX-PCR (Versalovic et al., 1991). Antibiotic resistance phenotypes and genotypes were determined for transconjugants and plasmid content was analyzed as described above.

3.9. Multilocus sequence typing

Multilocus sequence typing (MLST) was performed for selected isolates. PCR amplification and sequencing of seven housekeeping gene fragments (*adk*, *fumC*,

gyrB, *icd*, *mdh*, *purA* and *recA*) was performed following the protocols specified at the *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli) (Wirth et al., 2006). Sequences were edited and the allelic profile was searched against the MLST database to obtain the sequence type (ST).

3.10. Virulence factors screening

The presence of virulence genes associated with DEC strains pathotypes was determined using a multiplex PCR procedure for *stx* genes (*stx1* and *stx2*) and *eae* gene (Paton and Paton, 1998), and simplex PCR for *ipaH*, *aggR* and *elt* genes, adapted from a previous study (Aranda et al., 2007) (Table S2). Primers were used in different concentrations as follows: 250 nM for the multiplex assay (*stx1*, *stx2* and *eae*), 60 nM for *aggR*, 20 nM for *ipaH* and 5 nM for *elt* gene.

3.11. Nucleotide sequence accession numbers

Nucleotide sequences were deposited in GenBank database under the following accession numbers: KX579879-KX579882 (gene cassette arrays) and KX579883-KX579889 (bla_{TEM-1} genes from bacterial isolates).

4. RESULTS

4.1. Irrigation water quality

From each producer (n=16) the irrigation water quality was analyzed (Table S3). For all samples for which physical-chemical parameters were determined, at least one parameter deviated from recommended values (RV). pH values were below the RV for most samples, being even below the admissible values (AV) for three samples. Cl and NO_3 were also frequently above the RV (in 7 and 6 samples, respectively). Occasionally other parameters presented values above limits established by law (i.e. SO_4, As, Al, Mn and Fe). Two samples (Y4 and Y6) stood out in terms of low water quality, since values obtained for a high number of parameters were above (or below) the RV (5 parameters for Y4 and 6

parameters for Y6).

Total coliforms and enterococci are not included in Portuguese legislation for irrigation water quality evaluation (Ministério do Ambiente, 1998). However, high levels of total coliforms were detected for most samples while enterococci were detected in 9 out of 16 samples, with values ranging between 2 to 62 CFU/100mL.

4.2. *Escherichia coli* prevalence and diversity in water and vegetable samples

Escherichia coli were enumerable in 50% of water samples and in 38% of vegetable samples. However, *E. coli* counts in irrigation water were within the limits established by the Portuguese legislation (100 CFU/100 mL) (Ministério do Ambiente, 1998) varying between $7x10^{-2}$ to 21 CFU/100 mL, except for one sample (sample Y10; 304 *E. coli* CFU/100 mL). In vegetables, *E. coli* counting varied between $9x10^{-2}$ to 22 CFU/g (fresh weight), values that were within the limits established by the Portuguese law (100 CFU/g) (Comissão das Comunidades Europeias, 2005).

A total of 449 *E. coli* isolates were recovered from 8 water wells (n=210) and 7 vegetable samples (n=239) (Table S1). From these, 139 different BOX-PCR profiles were identified, of which 83 representative isolates were retrieved from irrigation water and 56 representative isolates were from vegetable samples (54 from lettuce, 1 from tomato and 1 from spinach).

Representative isolates were assigned to phylogenetic groups (A, B1, B2, C, D, E, F, and clade I). Overall, phylogroup B1 was the most prevalent (56% of isolates), followed by phylogroup A (22.3%) and phylogroup D (9.4%). Among isolates affiliated to phylogroup B1, 38 were from water (corresponding to 46% of the unique isolates from this source) and 40 (71%) from vegetable samples. Phylogroup A was detected mostly in isolates retrieved from water (26 isolates in contrast with 5 isolates from vegetables). Phylogroup E was detected in only 2 isolates from water and 1 from vegetables. Phylogroups C and D and clade I isolates were detected only in irrigation water (n=2 isolates, n=13 and n=1, respectively) while phylogroup B2 was detected only in vegetables (n=3).

4.3. Occurrence of diarrheagenic *E. coli* strains

Isolates were inspected for virulence determinants typical of DEC strains. Positive amplicons were obtained for genes *ipaH* (n=2 isolates) and *elt* (n=1) (Table 2). Positive isolates were all retrieved from vegetables, though from different samples.

4.4. Antibiotic susceptibility patterns in *E. coli* from water and vegetables

Resistance was detected to all antibiotics tested except to piperacillin/tazobactam (Table 1). One hundred and twenty-six *E. coli* isolates (91% of the total collection of unique isolates) were found to be resistant to one or more of the antibiotics tested. Resistance to streptomycin was the most common in isolates from both water and vegetables (89% resistant isolates in total; 86.7% and 93% resistant isolates from water and vegetables, respectively). Resistance to tetracycline was high for both sources (25% in total; 20.5 and 30.4% resistant isolates from water and vegetables, respectively), followed by amoxicillin/clavulanic acid (25.3 and 16.1% resistant isolates from water and vegetables, respectively). Isolates were more susceptible to aztreonam, cefotaxime, imipenem and chloramphenicol. For the majority of the antibiotics tested resistance rates were higher in isolates from vegetables (Table 1).

TABLE 1. Antibiotic resistance frequencies of *E. coli* unique isolates isolated from water (n=83) and vegetables (n=56).

Antibiotic[a]	No. of resistant isolates (%[b])	
	Water	**Vegetables**
AML	12 (14.5)	5 (8.9)
AMC	21 (25.3)	9 (16.1)
FEP	1 (1.2)	2 (3.6)
PRL	6 (7.2)	3 (5.3)
TZP	0 (0)	0 (0)
AZT	0 (0)	1 (1.8)
CAZ	3 (3.6)	3 (5.3)
CTX	1 (1.2)	0 (0)
IPM	2 (2.4)	0 (0)
CN	7 (8.4)	6 (10.7)
S	72 (86.7)	52 (93)
NA	4 (4.8)	2 (3.6)
CIP	1 (1.2)	2 (3.6)
TE	17 (20.5)	17 (30.4)
C	0 (0)	1 (1.8)
SXT	4 (4.8)	7 (12.5)

[a]AML- amoxicillin, AMC- amoxicillin/clavulanic acid, FEP- cefepime, PRL- piperacillin, TZP- piperacillin/tazobactam, AZT- aztreonam, CAZ- ceftazidime, CTX- cefotaxime, IMP- imipenem, CN- gentamicin, S- streptomycin, NA- nalidixic acid, CIP- ciprofloxacin, TE- tetracycline, C- chloramphenicol, SXT- sulfamethoxazole/trimethoprim.

[b]Percentage of resistant isolates of the total number of isolates obtained from each source.

4.5. Occurrence and characterization of ARGs, integrons and plasmids

Results from ARGs and integrons detection are presented in Table 2. The most commonly detected ARG was *tet*(B), in isolates from irrigation water (n=12, 15%) and vegetables (n=7, 13%). Other genetic determinants of resistance detected in both samples were: bla_{TEM} (identified as bla_{TEM-1} after amplicon sequencing), *tet*(A), *strA/strB* and *sul2*. Genes *sul1*, *dfrA1*, *aadA1* and *aadA2* were only detected in isolates from water. Quinolone resistance determinants were not detected, except for a mutation on the *gyrA* gene (S83L) in an isolate from water, with low susceptibility to nalidixic acid and ciprofloxacin. Genes encoding resistance to carbapenems and 3rd generation cephalosporins were not detected.

Class 1 integrons were present in five strains, two of which carrying the arrays *dfrA1-aadA1* and *dfrA16-*bla_{P1b}*-aadA2-ereA*. A class 2 integron was detected in one isolate from water carrying the array *dfrA1-sat2-aadA1*.

Plasmid DNA was purified from 14 isolates, 10 of which displayed an MDR phenotype (see section below and Table 2). Replicon typing revealed the presence of IncI1, IncFIB, IncFIC, IncFrep and IncY replicons. IncFrep was found associated to most of the plasmid-positive isolates (8/14) retrieved both from water and vegetable

4.6. Characterization of MDR strains

Twenty-two isolates (16% of the total collection of unique isolates) were found to be MDR (i.e., resistant to at least three different antibiotic classes). Multidrug resistance level was higher for isolates from vegetables (21% of the isolates) than from water (12%) (Table 2). Streptomycin resistance was present in 100% of the MDR strains, followed by tetracycline (82%) and sulfamethoxazole/trimethoprim (50%). MDR isolates were resistant against 3 to 9 different antibiotics. Most MDR isolates carried ARGs, five isolates carried a class 1 integron and 1 isolate carried a class 2 integron (Table 2).

Plasmid DNA was purified from 10 MDR isolates. IncI1, IncFIB, IncFIC and IncFrep replicons were detected. The most prevalent replicon was IncFrep that was found associated to most of the MDR plasmid-positive isolates (6/10),

TABLE 2. Phenotypes and genotypes for MDR strains (in bold) and other representative isolates that gave a positive result in at least one PCR experiment.

Donor									Transconjugant		
Isolate	Origin[a]	PG	MLST	AR Phenotype[b]	AR/Virulence Genotype	Integron (array)	pDNA replicons	Conjugation assays	AR Phenotype[b]	ARGs	Integron
Y5W.8	W/L	A	n.d.	S-TE	*tet*(A)	-	-	n.d.	n.d.	n.d.	n.d.
Y9W.1	**W**	**E**	**unk.**	**S-TE-SXT**	***tet*(A)-*sul1*-*aadA1*-*dfrA1***	***IntI1* (*dfrA1*-*aadA1*)**	**I1**	**+**	**TE-SXT**	***tet*(A)-*sul1*-*aadA1*-*dfrA1***	**+**
Y10W.9	**W**	**D**	**ST297**	**AML-PRL-S-TE**	***bla*$_{TEM-1}$-*tet*(A)-*tet*(B)**	**-**	**-**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
Y10W.25	W	B1	n.d.	S-TE	*tet*(B)-*strA/B*	-	-	n.d.	n.d.	n.d.	n.d.
Y10W.43	W	D	n.d.	S-TE	*tet*(B)-*strA/B*	-	-	n.d.	n.d.	n.d.	n.d.
Y10W.70	W	A	n.d.	S-TE	*tet*(B)	-	-	n.d.	n.d.	n.d.	n.d.
Y10W.75	W	B1	n.d.	S-TE	*tet*(B)-*strA/B*	-	-	n.d.	n.d.	n.d.	n.d.
Y10W.88	**W**	**D**	**ST297**	**AML-PRL-S-TE**	***bla*$_{TEM-1}$-*tet*(B)**	**-**	**-**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
Y15W.2	W	A	n.d.	S-TE	*tet*(A)	-	-	n.d.	n.d.	n.d.	n.d.
Y15W.3	W	A	n.d.	AML-AMC-PRL-CN-S	*bla*$_{TEM-1}$-*strA/B*	-	-	n.d.	n.d.	n.d.	n.d.
Y15W2.1	**W**	**A**	**ST10**	**AML-AMC-PRL-S-TE**	***bla*$_{TEM-1}$-*tet*(B)-*strA/B***	**-**	**FIB-Frep**	**-**	**n.d.**	**n.d.**	**n.d.**
Y15W2.3	**W**	**D**	**ST297**	**AMC-S-TE**	***tet*(B)**	**-**	**FIC**	**+**	**TE**	***tet*(B)**	**-**
Y15W2.4	**W**	**A**	**ST10**	**S-TE-SXT**	***tet*(B)-*aadA1*-*dfrA1***	***IntI2* (*dfrA1*-*sat2*-*aadA1*)**	**Frep**	**+**	**S-TE**	***tet*(B)-*aadA1*-*dfrA1***	**+**
Y16W.3	W	A	n.d.	S-TE	*tet*(B)	-	-	n.d.	n.d.	n.d.	n.d.
Y16W2.1	**W**	**D**	**ST38**	**AML-AMC-PRL-S-SXT**	***aadA2*-*strA/B***	***IntI1* (*dfrA16*-*blaP1b*-*aadA2*-*ereA1*)**	**-**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
Y16W2.2	**W**	**B1**	**ST101**	**AML-AMC-PRL-CN-S-SXT**	***bla*$_{TEM-1}$-*sul2*-*strA/B***	***IntI1* (n.d.)**	**-**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
Y16W2.3	W	A	n.d.	AMC-S	*straA/B*	-	-	n.d.	n.d.	n.d.	n.d.
Y16W2.7	W	Clade I	n.d.	CN-S-TE	*tet*(B)	-	-	n.d.	n.d.	n.d.	n.d.
Y16W2.17	**W**	**C**	**ST88**	**AMC-S-TE**	***tet*(B)**	**-**	**-**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
Y16W2.28	**W**	**D**	**ST212**	**AMC-S-NA-CIP**	***gyrA* (S83L)**	**-**	**Frep**	**-**	**n.d.**	**n.d.**	**n.d.**

Y7V.3	**L**	**B1**	**ST446**	**AMC-S-TE**	**-**	**-**	**-**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
Y10V.1	L	B1	n.d.	S	*-/elt*	-	-	n.d.	n.d.	n.d.	n.d.
Y10V.44	L	MLST	n.d.	S-TE	*tet*(B)	-	-	n.d.	n.d.	n.d.	n.d.
Y12V.15	L	B2	n.d.	CN-S-TE	*tet*(B)	-	-	n.d.	n.d.	n.d.	n.d.
Y12V.18	**L**	**B2**	**ST10**	**PRL-S-TE**	***bla*$_{TEM-1}$-*tet*(B)-*strA/B***	**-**	**FIB-Frep**	**+**	**AML-PRL-TE**	***bla*$_{TEM}$-*tet*(B)-*strA/B***	**-**
Y15V.4	**L**	**E**	**unk.**	**AML-AMC-PRL-S-TE-C**	***bla*$_{TEM-1}$-*tet*(A)-*strA/B***	**-**	**I1-Frep**	**+**	**AMC-AML-PRL-S-TE-C**	***bla*$_{TEM}$-*tet*(A)-*strA/B***	**-**
Y15V.7	**L**	**B1**	**ST2522**	**S-TE-SXT**	***tet*(B)-*sul2*-*strA/B***	**-**	**-**	n.d.	**n.d**	**n.d**	**n.d**
Y15V.16	**L**	**B1**	**ST2522**	**AMC-S-TE-SXT**	***tet*(B)-*sul2*-*strA/B***	**-**	**-**	n.d.	**n.d**	**n.d**	**n.d**
Y15V.22	**W/L**	**A**	**ST48**	**S-TE-SXT**	***tet*(A)-*sul2***	***IntI1* (n.d.)**	**-**	n.d.	**n.d**	**n.d**	**n.d**
Y15V.41	**L**	**A**	**ST48**	**S-TE-SXT**	***tet*(A)-*sul2***	**-**	**-**	n.d.	**n.d**	**n.d**	**n.d**
Y15V.42	L	MLST	n.d.	S-TE	*tet*(B)-*strA/B*	-	-	n.d.	n.d.	n.d.	n.d.
Y15V.50	**L**	**B1**	**ST2522**	**S-TE-SXT**	***tet*(B)-*sul2*-*strA/B***	**-**	**-**	n.d.	**n.d**	**n.d**	**n.d**
Y15V.52	**L**	**B1**	**ST424**	**AML-AMC-S-TE-SXT**	**-**	**-**	**unk.**	**-**	**n.d**	**n.d**	**n.d**
Y15V.54	**L**	**A**	**unk.**	**S-TE-SXT**	***tet*(A)-*sul2***	***IntI1* (n.d.)**	**unk.**	**+**	**AMC-AML-PRL-S-TE-C-TZP**	***tet*(A)-*sul2***	**-**
Y15V.65	L	MLST	n.d.	S	*-/ipaH*	-	-	n.d.	n.d.	n.d.	n.d.
Y15V.95	L	B1	n.d.	S	*-/ipaH*	-	-	n.d.	n.d.	n.d.	n.d.
Y15V.97	**L**	**B1**	**ST424**	**AMC-S-NA**	**-**	**-**	**-**	n.d.	**n.d.**	**n.d.**	**n.d.**
Y16V.5	**L**	**B1**	**ST155**	**AML-FEP-PRL-AZT-CAZ-CN-S-CIP-TE**	***bla*$_{TEM-1}$-*tet*(A)**	**-**	**FIB-Frep**	**+**	**AMC-AML-PRL-TE**	***bla*$_{TEM}$-*tet*(A)**	**-**

[a]W, water; L, lettuce.
PG, Phylogenetic group.
unk., unknown.
[b]AML- amoxicillin, AMC- amoxicillin/clavulanic acid, FEP- cefepime, PRL- piperacillin, TZP- piperacillin/tazobactam, AZT- aztreonam, CAZ- ceftazidime, CTX- cefotaxime, IMP- imipenem, CN- gentamicin, S- streptomycin, NA- nalidixic acid, CIP- ciprofloxacin, TE- tetracycline, C- chloramphenicol, SXT- sulfamethoxazole/trimethoprim.
n.d., not determined; +, positive result; -, negative result.

retrieved from both water and vegetables. Conjugation experiments were conducted in order to assess the motility potential of the plasmids. For this, we selected transconjugants in medium supplemented with tetracycline or streptomycin, which corresponded to frequent resistance phenotypes among plasmid-positive isolates. Transfer of these phenotypes was successful for 7 *E. coli* isolates. All transconjugants received the AR determinants and two of them received the integrons detected in the donor strains (Table 2).

MDR isolates were assigned to eleven discrete STs (Table 2). From these, most were exclusive from water (5 STs) or vegetables (5 STs). One of the isolates identified as ST48 corresponded to an isolate collected in both sources (clonal group C; see section 4.7). ST10 was found in isolates from both water and vegetable samples (2 and 1 isolates, respectively).

4.7. Occurrence of the same *E. coli* clones in water and vegetable samples

To confirm the presence of identical isolates in water and vegetables, clonality of isolates displaying identical BOX profiles was further checked using REP- and ERIC-PCR. From this analysis, 7 clonal groups were detected that included isolates from water and vegetable samples (Table 3). In some cases, clonal isolates were retrieved from two producers (clonal groups B, C, D, E, F and G) or three producers (clonal group A). Isolates included in clonal group A were the most frequently retrieved (3 isolates from water and 6 from vegetables), followed by isolates from clonal group D (1 isolate from water and 7 from vegetables). An isolate representative of each clonal group was selected for further analysis (Table 3). Isolates belong to ST48 (clonal group C), ST1081 (clonal group A), ST1432 (clonal group D), ST2313 (clonal group E) and ST2308 (clonal group G). Clonal groups B and F were assigned as unknown STs. Multidrug resistance was detected in isolates representing clonal group C, one of which carried the *tet(A)* and *sul2* genes and a class 1 integron with undetermined array. Plasmids were detected in isolates representing 3 clonal groups (A, B and G) and plasmid typing revealed the presence of FIC, FREP, FIB and Y replicons. None of the plasmids were transferable to *E. coli* CV601.

TABLE 3. Characteristics of isolates representing clonal groups isolated both from water and vegetable samples.

Clonal group[a]	Number of isolates from water	Number of isolates from vegetables	Representative isolate	PG	MLST	AR phenotype	AR genotype	Integron (array)	Plasmid	pDNA replicons	Conjugation assays
A	3	6	Y15V.38	B1	ST1081	AMC-S	-	-	+	FIC-Y	-
B	1	5	Y15V.30	B1	unk.	AMC-CAZ-CN-S	-	-	+	FIC-Frep	-
C	1	1	Y15V.22	A	ST48	S-TE-SXT	*tet*(A)-*sul2*	*Intl1*(n.d.)	-	-	n.d
D	1	7	Y4W.26	B1	ST1432	AML-AMC-IPM	-	-	-	-	n.d
E	1	5	Y16W.1	B1	ST2313	AMC-S	-	-	-	-	n.d
F	2	1	Y15V.79	B1	unk.	S	-	-	-	-	n.d
G	1	4	Y15W.1	B1	ST2308	AMC-S	-	-	+	FIC-FIB-Frep	-

[a]according to rep-PCR typing (BOX-, ERIC- and REP-PCR).
PG, Phylogenetic group.
unk., unknown.
n.d., not determined; +, positive result; -, negative result.

5. DISCUSSION

In this study, we analyzed *E. coli* present in groundwater used for irrigation and in raw-eaten vegetables from domestic producers. Isolates were characterized in terms of antibiotic resistance phenotypes and genotypes, and virulence potential to get insight into the potential risk for human health. The possible role of irrigation water as a route of contamination of fresh produce was assessed.

5.1. Antibiotic resistance traits in *E. coli* from irrigation water and vegetables

Resistance to all classes of antibiotics was detected in both water and vegetables. Irrespective of the source, high antibiotic resistance prevalence was observed for streptomycin and tetracycline followed by resistance towards penicillins (AMC and AML) and the combination SXT. High resistance levels to these antibiotics were previously reported in *E. coli* from different aquatic systems (Pereira et al., 2013) and lettuce (Holvoet et al., 2013). Streptomycin and tetracycline are ancient antibiotics widely used in different non-clinical settings (European Center for Disease Prevention and Control et al., 2015). In fact, according to a recently published report, tetracyclines were the most consumed antibiotics in food-producing animals (55.5 tonnes) in Portugal in 2012 (European Center for Disease Prevention and Control et al., 2015).

Most relevant, we detected resistance to antibiotics that are critically important to human health and to which resistance is rare in *E. coli* from water (Alves et al., 2014; Tacão et al., 2014) or vegetables (Holvoet et al., 2013). For example, 2.2% of the total number of isolates analyzed in this study (1 and 2 isolates from water and vegetables, respectively), were resistant to ciprofloxacin and 9.4% were resistant to gentamicin (7 and 6 isolates from water and vegetables, respectively) (Table 1). In this geographic area, previous studies concluded that surface water was polluted with ARGs and ARB with high clinical relevance, that persist due to continuous anthropogenic selective pressure (Tacão

et al., 2015, 2012).

Multidrug resistance was detected among strains from both water and vegetable samples with levels being even higher among isolates from vegetables. Our results could be attributed to other sources of vegetables contamination contributing with MDR strains, such as the use of organic fertilizers, a common practice in Portugal, and particularly in domestic production. Fertilization with manure has been pointed as a relevant source of ARGs and/or ARB to soils and vegetables (Marti et al., 2013).

All integron-positive isolates were MDR (Table 2), highlighting the role of these genetic platforms in the dispersion of MDR traits. Integrons carried different arrays with genes conferring resistance to several antibiotics largely used in human-medicine (Direção-Geral da Saúde, 2014; European Center for Disease Prevention and Control et al., 2015). The detected arrays *dfrA1-aadA1* and *dfrA1-SAT2-aadA1* were previously found in a variety of microorganisms (including *E. coli*) and different settings (Integrall database - Moura et al., 2009), including aquatic systems (Laroche et al., 2009; Tacão et al., 2014) and wastewater in Portugal (Moura et al., 2007).

The dissemination potential of ARGs detected in MDR *E. coli* was further confirmed in mating assays. Plasmids encoding tetracycline resistance were frequently transferable to a receptor strain. These plasmids carried resistance genes conferring other resistance phenotypes to the receptor strain, in some cases to three or more classes of antibiotics (e.g. isolates Y15 V.4 and Y15 V.54). As in other studies, these results confirm the importance of conjugative plasmids in the dissemination of antibiotic resistance (Bennett, 2009).

To our knowledge it is the first time that IncFIC is found in irrigation water (Y15 W.1 and Y15 W2.3) and vegetables (Y15 V.30 and Y15 V.38) (in this study found in water and lettuce from the same producer and as clonal isolates found among several producers; see section 4.3). This replicon has been found in pathogenic *E. coli* isolated from animal (Chah et al., 2010; Jahanbakhsh et al., 2016) and human sources (Chah et al., 2010; Moran et al., 2015), frequently displaying multidrug resistance and carrying relevant ARGs such as plasmidic bla_{CMY-2} (Shahada et al., 2013).

5.2. Virulence potential of *E. coli* isolates

Phylogenetic distribution has been pointed as an indication of the virulence potential of *E. coli* (Mosquito et al., 2015). Recent studies relate *E. coli* strains of phylotypes A and B1 with intestinal or extra-intestinal infections (Rodrigues et al., 2015; Valverde et al., 2009), suggesting their potential to spread to humans via food chain. Strains from phylotypes A and B1 were highly prevalent in both water and vegetable samples. Besides, 2 enteroinvasive *E. coli* (EIEC) strains in vegetables from the same producer (Y15 V.65 and Y15 V.95) and one enterotoxigenic *E. coli* (ETEC) strain also from vegetable samples (Y10 V.1) were identified (Table 2). As ETEC is frequently recognized as a waterborne pathogen (Ahmed et al., 2013), and less commonly as a foodborne pathogen, the origin of these strains may be irrigation water.

Most MDR isolates were assigned to STs which belong to largely widespread STs frequently associated to infections worldwide (e.g. ST10, ST38 and ST101) and that were reported to carry genes encoding important widespread β-lactamases, including ESBLs and carbapenemases (e.g. CTX-M and OXA-48-like) (A. Potron et al., 2013). Strains belonging to ST10 clonal complex (in this study ST10 and ST48) have been detected in avian pathogenic *E. coli*, and associated to poultry and human cases of urinary tract infections and sepsis which pose a potential zoonotic risk (Giufrè et al., 2012). D:ST38 has been already found in vegetable samples belonging to the diarrheagenic enteroaggregative *E. coli* (EAEC) D:ST38 lineage (Zurfluh et al., 2015). This ST has also been implicated in extraintestinal pathogenic *E. coli* (ExPEC) infections (Chattaway et al., 2014). Worldwide spread of ST101shiga toxin-producing *E. coli* (STEC) strains has been reported (Koo et al., 2012) and related with infections in pediatric clinical settings and with carbapenem resistance (namely production of the NDM-1 beta-lactamase) (Pannaraj et al., 2015).

5.3. Role of irrigation water as a source of vegetables contamination

Values for irrigation water quality parameters were below or above those recommended by law (Ministério do Ambiente, 1998) indicating that generally the water quality in wells in this geographic area was poor. Besides the intensive industrial activity in this area (nearby the second largest chemical industry

complex in Portugal producing mostly chlorides, nitrates and synthetic resins), other factors may contribute to groundwater contamination such as runoffs from agricultural fields (Miraldo, 2007). The comparison to other geographic regions in Portugal was not possible since, to our knowledge, this is the first study to provide information about the microbiological quality of groundwater used to irrigate fresh produce in the country.

Escherichia coli was detected in 50% of the water samples, a percentage higher than the ones reported in private water wells for other European countries (Richardson et al., 2009). Also more frequently than previously reported (Abadias et al., 2008; Amézquita-Montes et al., 2015; Boehme et al., 2004; Hassan et al., 2011; Osterblad et al., 1999; Schwaiger et al., 2011; Viswanathan and Kaur, 2001), vegetables of 6 different producers were contaminated with *E. coli* (38% of the vegetable samples). It is important to highlight the fact that frequently the producers for whom irrigation water contamination with *E. coli* was identified were the same for which contamination of vegetables was detected. In this study, as other studies, higher levels of contamination were consistently detected in leafy green vegetables, probably due to a larger surface area that provides conditions for microorganisms to attach and survive (De Roever, 1999).

The presence of the same clones in irrigation water and vegetables was confirmed by rep-PCR (BOX-, REP- and ERIC-PCR). As stated by other authors (Araújo et al., 2014), isolates displaying identical profiles when using these three methodologies are genotypically identical. This was true for 7 different clonal groups of isolates, further confirming the contribution of irrigation water as a source of vegetables contamination with *E. coli*. All clonal groups were found in more than one farm. In three of the clones (clonal groups A, B and G) more than one conjugative plasmids was detected (Table 3). IncFIC replicon, an extremely rare replicon in the environment, was found among these three clonal groups, and was spread through three different farms, both in irrigation water and vegetable samples. MDR clonal group C belonging to A:ST48 carrying a class I integron and the representative isolate of clonal group G assigned to ST2308, previously found in a CTX-M-8-producing *E. coli* strain in buffalo feces (Aizawa et al., 2014), are all evidences that reinforce the burden of the dissemination of antibiotic resistance in the environment, that happens mostly through mobile genetic elements exchange (Von Wintersdorff et al., 2016).

6. CONCLUSIONS

This is the first report on the microbiological quality of fresh vegetables collected from household farms in Portugal. Diverse risk factors were identified in these strains, namely the fact that were frequently MDR and carried acquired resistance genes transferable to a different host through conjugation. Also the prevalent phylogenetic groups in both sources have been commonly associated to DEC strains and occasionally virulence genes were detected in these isolates. STs frequently found in clinics and associated to clinically relevant ARGs were detected. Evidences were found that contaminated irrigation water constitutes a source of *E. coli* that may enter the food chain through the consumption of raw vegetables. In most cases, water and vegetables contamination were detected in the same farm. Besides, we detected the same rep-PCR genotypes and the same STs in both vegetables and water, occasionally widespread among several producers. The results presented here are worrying and draw attention to a problem that has been overlooked: the quality of vegetables produced in small household farms. This study highlight the need for regular monitoring of these food products, ideally also assessing parameters related to antibiotic resistance. Irrigation water should also be monitored considering these parameters. The poor water quality verified in the analyzed farms justifies *per se* the need to identify the sources of contamination and to protect the wells in use. Finally, the role of irrigation water as a source of contamination should be assessed in future studies in other geographical regions.

7. ACKNOWLEDGMENTS

Authors gratefully acknowledge Dr. Alessandra Carattoli for the positive controls for detection of plasmid replicons, Dr. Josée Harel from Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, QC, Canada and Dr. Björn Posse from Faculty of Veterinary Medicine, University of Ghent, Belgium for the positive controls for detection of DEC strains.

We also thank Marta Alves for the support during sampling. This work was supported by National Funds from FCT – Fundação para a Ciência e a Tecnologia

- through project WaterJPI/0002/2013 "StARE: Stopping Antibiotic Resistance Evolution". Authors also acknowledge FCT financing to CESAM (UID/AMB/50017/2013–POCI-01-0145-FEDER-007638) and IBiMED (UID/BIM/04501/2013), Artur Alves (FCT Investigator Programme – IF/00492/2013), Isabel Henriques (FCT Investigator Programme – IF/00492/2013), Marta Tacão (Pos-doc grant SFRH/BPD/114855/2016) and Susana Araújo (PhD grant SFRH/BD/52573/2014). The authors would like to acknowledge the COST-European Cooperation in Science and Technology, through COST Action ES1403: New and emerging challenges and opportunities in wastewater reuse (NEREUS).

8. REFERENCES

Abadias, M., Usall, J., Anguera, M., Solsona, C., Viñas, I., 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. Int. J. Food Microbiol. 123, 121–129. doi:10.1016/j.ijfoodmicro.2007.12.013

Ahmed, D., Islam, M.S., Begum, Y.A., Janzon, A., Qadri, F., Sjöling, Å., 2013. Presence of enterotoxigenic *Escherichia coli* in biofilms formed in water containers in poor households coincides with epidemic seasons in Dhaka. J. Appl. Microbiol. 114, 1223–1229. doi:10.1111/jam.12109

Aizawa, J., Neuwirt, N., Barbato, L., Neves, P.R., Leigue, L., Padilha, J., Pestana de Castro, A.F., Gregory, L., Lincopan, N., 2014. Identification of fluoroquinolone-resistant extended-spectrum β-lactamase (CTX-M-8)-producing *Escherichia coli* ST224, ST2179 and ST2308 in buffalo (*Bubalus bubalis*). J. Antimicrob. Chemother. 69, 2866–2869. doi:10.1093/jac/dku218

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402. doi:10.1093/nar/25.17.3389

Alves, M.S., Pereira, A., Araújo, S.M., Castro, B.B., Correia, A.C.M., Henriques, I., 2014. Seawater is a reservoir of multi-resistant *Escherichia coli*, including strains hosting plasmid-mediated quinolones resistance and extended-spectrum beta-lactamases genes. Front. Microbiol. 5, 1–10.

doi:10.3389/fmicb.2014.00426

Amézquita-Montes, Z., Tamborski, M., Kopsombut, U.G., Zhang, C., Arzuza, O.S., Gómez-Duarte, O.G., 2015. Genetic relatedness among *Escherichia coli* pathotypes isolated from food products for human consumption in Cartagena, Colombia. Foodborne Pathog. Dis. 12, 454–461. doi:10.1089/fpd.2014.1881

Aranda, K.R.S., Fabbricotti, S.H., Fagundes-Neto, U., Scaletsky, I.C.A., 2007. Single multiplex assay to identify simultaneously enteropathogenic, enteroaggregative, enterotoxigenic, enteroinvasive and Shiga toxin-producing *Escherichia coli* strains in Brazilian children. FEMS Microbiol. Lett. 267, 145–150. doi:10.1111/j.1574-6968.2006.00580.x

Araújo, S., Henriques, I.S., Leandro, S.M., Alves, A., Pereira, A., Correia, A., 2014. Gulls identified as major source of fecal pollution in coastal waters: A microbial source tracking study. Sci. Total Environ. 470–471, 84–91. doi:10.1016/j.scitotenv.2013.09.075

Bennett, P.M., 2009. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. Br. J. Pharmacol. 153, S347–S357. doi:10.1038/sj.bjp.0707607

Berger, C.N., Sodha, S. V, Shaw, R.K., Griffin, P.M., Pink, D., Hand, P., Frankel, G., 2010. Fresh fruit and vegetables as vehicles for the transmission of human pathogens. Environ. Microbiol. doi:10.1111/j.1462-2920.2010.02297.x

Beuchat, L.R., 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. Microbes Infect. doi:10.1016/S1286-4579(02)01555-1

Beuchat, L.R., 1996. Pathogenic Microorganisms Associated with Fresh Produce. J. Food Prot. 2, 204–216.

Beuchat, L.R., Ryu, J.H., 1997. Produce Handling and Processing Practices. Emerg. Infect. Dis. 3, 459–465. doi:10.3201/eid0304.970407

Biological Hazards (BIOHAZ) Panel, Andreoletti, O., Baggesen, D.L., Bolton, D., Butaye, P., Cook, P., Davies, R., Escámez, P.S.F., Griffin, J., Hald, T., Havelaar, A., Koutsoumanis, K., Lindqvist, R., McLauchlin, J., Nesbakken, T., Prieto M, M., Sofos, J., Threlfall, J., 2013. Scientific opinion on the risk posed by pathogens in food of non-animal origin . Part 1 (outbreak data analysis and risk ranking of food/pathogen combinations), EFSA Journal.

doi:10.2903/j.efsa.2013.3025.

Boehme, S., Werner, G., Klare, I., Reissbrodt, R., Witte, W., 2004. Occurrence of antibiotic-resistant enterobacteria in agricultural foodstuffs. Mol. Nutr. Food Res. 48, 522–531. doi:10.1002/mnfr.200400030

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K.L., Threlfall, E.J., 2005. Identification of plasmids by PCR-based replicon typing. J. Microbiol. Methods 63, 219–228. doi:10.1016/j.mimet.2005.03.018

Chah, K.F., Agbo, I.C., Eze, D.C., Somalo, S., Estepa, V., Torres, C., 2010. Antimicrobial resistance, integrons and plasmid replicon typing in multiresistant clinical *Escherichia coli* strains from Enugu State, Nigeria. J. Basic Microbiol. 50, 18–24. doi:10.1002/jobm.200900325

Chattaway, M.A., Jenkins, C., Ciesielczuk, H., Day, M., DoNascimento, V., Day, M., Rodríguez, I., van Essen-Zandbergen, A., Schink, A.-K., Wu, G., Threlfall, J., Woodward, M.J., Coldham, N., Kadlec, K., Schwarz, S., Dierikx, C., Guerra, B., Helmuth, R., Mevius, D., Woodford, N., Wain, J., 2014. Evidence of evolving extraintestinal enteroaggregative *Escherichia coli* ST38 clone. Emerg. Infect. Dis. 20, 1935–1937. doi:10.3201/eid2011.131845

Clermont, O., Christenson, J.K., Denamur, E., Gordon, D.M., 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ. Microbiol. Rep. 5, 58–65. doi:10.1111/1758-2229.12019

Comissão das Comunidades Europeias, 2005. Regulamento (CE) No. 2073/2005 da comissão de 15 de Novembro de 2005 relativo a critérios microbiológicos aplicáveis a géneros alimentícios. J. Of. da União Eur.

De Roever, C., 1999. Microbiological safety evaluations and recommendations on fresh produce. Food Control 10, 117–143. doi:10.1016/S0956-7135(99)00026-2

Direção-Geral da Saúde, 2014. Prevenção e Controlo de Infeções e de Resistência aos Antimicrobianos em números – 2014.

Direção-Geral De Agricultura, 2003. Situação da Agricultura em Portugal, Comissão Europeia.

Edelstein, M., Sundborger, C., Hergens, M., Ivarsson, S., Dryselius, R., Insulander, M., Jernberg, C., Hutin, Y., Wallensten, A., 2014. Barriers to trace-back in a

salad-associated EHEC outbreak, Sweden, June 2013. PLOS Curr. Outbreaks 1–20. doi:10.1371/currents.outbreaks.80bbab3af3232be0372ea0e904dcd1fe

Elmadfa, I., Meyer, A., Nowak, V., Hasenegger, V., Putz, P., Verstraeten, R., Remaut-DeWinter, A.M., Kolsteren, P., Dostálová, J., Dlouhý, P., Trolle, E., Fagt, S., Biltoft-Jensen, A., Mathiessen, J., Velsing Groth, M., Kambek, L., Gluskova, N., Voutilainen, N., Erkkilä, A., Vernay, M., Krems, C., Strassburg, A., Vasquez-Caicedo, A.L., Urban, C., Naska, A., Efstathopoulou, E., Oikonomou, E., Tsiotas, K., Bountziouka, V., Benetou, V., Trichopoulou, A., Zajkás, G., Kovács, V., Martos, E., Heavey, P., Kelleher, C., Kennedy, J., Turrini, A., Selga, G., Sauka, M., Petkeviciene, J., Klumbiene, J., Holm Totland, T., Andersen, L.F., Halicka, E., Rejman, K., Kowrygo, B., Rodrigues, S., Pinhão, S., Ferreira, L.S., Lopes, C., Ramos, E., Vaz Almeida, M.D., Vlad, M., Simcic, M., Podgrajsek, K., Serra Majem, L., Román Viñas, B., Ngo, J., Ribas Barba, L., Becker, V., Fransen, H., Van Rossum, C., Ocké, M., Margetts, B., 2009. European Nutrition and Health Report 2009., Forum of nutrition. 62, 1–405. doi:10.1159/000242367

European Center for Disease Prevention and Control, European Food Safety Authority, European Medicines Agency, 2015. ECDC/EFSA/EMA first joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals, EFSA Journal. doi:10.2903/j.efsa.2015.4006

European Commission, 2008. Groundwater Protection in Europe. The New Groundwater Directive-Consolidating the EU Regulatory Framework. doi:10.2779/84304

European Community, 2000. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy, Official Journal of the European Parliament. doi:10.1039/ap9842100196

FAO/WHO, 2008. Microbiological hazards in fresh leafy vegetables and herbs. doi:987-92-5-106118-3

Friesema, I., Sigmundsdottir, G., van der Zwaluw, K., Heuvelink, A., Schimmer, B., de Jager, C., Rump, B., Briem, H., Hardardottir, H., Atladottir, A., Gudmundsdottir, E., van Pelt, W., 2008. An international outbreak of Shiga toxin-producing *Escherichia coli* O157 infection due to lettuce, September - October 2007. Eurosurveillance 13, 1–5.

Giufrè, M., Graziani, C., Accogli, M., Luzzi, I., Busani, L., Cerquetti, M., 2012. *Escherichia coli* of human and avian origin: detection of clonal groups associated with fluoroquinolone and multidrug resistance in Italy. J. Antimicrob. Chemother. 67, 860–867. doi:10.1093/jac/dkr565

Hamilton-Miller, J.M.., Shah, S., 2001. Identity and antibiotic susceptibility of enterobacterial flora of salad vegetables. Int. J. Antimicrob. Agents 18, 81–83. doi:10.1016/S0924-8579(01)00353-3

Hassan, S.A., Altalhi, A.D., Gherbawy, Y.A., El-Deeb, B.A., 2011. Bacterial load of fresh vegetables and their resistance to the currently used antibiotics in Saudi Arabia. Foodborne Pathog. Dis. 8, 1011–1018. doi:10.1089/fpd.2010.0805

Henriques, I.S., Fonseca, F., Alves, A., Saavedra, M.J., Correia, A., 2006. Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from estuarine waters. Res. Microbiol. 157, 938–947. doi:10.1016/j.resmic.2006.09.003

Hilborn, E.D., Mermin, J.H., Mshar, P.A., Hadler, J.L., Voetsch, A., Wojtkunski, C., Swartz, M., Mshar, R., Lambert-Fair, M.-A., Farrar, J.A., Glynn, M.K., Slutsker, L., 1999. A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of Mesclun Lettuce. Arch. Intern. Med. 159, 1758. doi:10.1001/archinte.159.15.1758

Holvoet, K., Sampers, I., Callens, B., Dewulf, J., Uyttendaele, M., 2013. Moderate Prevalence of antimicrobial resistance in *Escherichia coli* isolates from lettuce, irrigation water, and soil. Appl. Environ. Microbiol. 79, 6677–6683. doi:10.1128/AEM.01995-13

Jahanbakhsh, S., Smith, M.G., Kohan-Ghadr, H.-R., Letellier, A., Abraham, S., Trott, D.J., Fairbrother, J.M., 2016. Dynamics of extended-spectrum cephalosporin resistance in pathogenic *Escherichia coli* isolated from diseased pigs in Quebec, Canada. Int. J. Antimicrob. Agents. 48, 194–202. doi:10.1016/j.ijantimicag.2016.05.001

Jung, Y., Jang, H., Matthews, K.R., 2014. Effect of the food production chain from farm practices to vegetable processing on outbreak incidence. Microb. Biotechnol. 7, 517–527. doi:10.1111/1751-7915.12178

Kaper, J.B., Nataro, J.P., Mobley, H.L.T., 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. 2, 123–140. doi:10.1038/nrmicro818

Koo, H.J., Kwak, H.S., Yoon, S.H., Woo, G.J., 2012. Phylogenetic group

distribution and prevalence of virulence genes in *Escherichia coli* isolates from food samples in South Korea. World J. Microbiol. Biotechnol. 28, 1813–1816. doi:10.1007/s11274-011-0954-5

Laroche, E., Pawlak, B., Berthe, T., Skurnik, D., Petit, F., 2009. Occurrence of antibiotic resistance and class 1, 2 and 3 integrons in *Escherichia coli* isolated from a densely populated estuary (Seine, France). FEMS Microbiol. Ecol. 68, 118–130. doi:10.1111/j.1574-6941.2009.00655.x

LeJeune, J.T.L.E., Besser, T.E., Hancock, D.D., 2001. Cattle Water Troughs as Reservoirs of Escherichia coli O157. Appl. Environ. Microbiol. 67, 3053–3057. doi:10.1128/AEM.67.7.3053

Lupo, A., Coyne, S., Berendonk, T.U., 2012. Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. Front. Microbiol. 3, 18. doi:10.3389/fmicb.2012.00018

Marti, R., Scott, A., Tien, Y.-C., Murray, R., Sabourin, L., Zhang, Y., Topp, E., 2013. Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. Appl. Environ. Microbiol. 79, 5701–5709. doi:10.1128/AEM.01682-13

Ministério do Ambiente, 1998. Decreto-Lei n.º 236/1998 de 1 de Agosto, Diário da República.

Miraldo, C.M., 2007. Estudo da contaminação do aquífero superior na região de Estarreja. Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Moran, R.A., Anantham, S., Pinyon, J.L., Hall, R.M., 2015. Plasmids in antibiotic susceptible and antibiotic resistant commensal *Escherichia coli* from healthy Australian adults. Plasmid 80, 24–31. doi:10.1016/j.plasmid.2015.03.005

Mosquito, S., Pons, M.J., Riveros, M., Ruiz, J., Ochoa, T.J., 2015. Diarrheagenic *Escherichia coli* phylogroups are associated with antibiotic resistance and duration of diarrheal episode. Sci. World J. 2015, 1–6. doi:10.1155/2015/610403

Moura, A., Henriques, I., Ribeiro, R., Correia, A., 2007. Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. J. Antimicrob. Chemother. 60, 1243–1250. doi:10.1093/jac/dkm340

Moura, A., Oliveira, C., Henriques, I., Smalla, K., Correia, A., 2012a. Broad diversity of conjugative plasmids in integron-carrying bacteria from wastewater environments. FEMS Microbiol. Lett. 330, 157–164. doi:10.1111/j.1574-6968.2012.02544.x

Moura, A., Pereira, C., Henriques, I., Correia, A., 2012b. Novel gene cassettes and integrons in antibiotic-resistant bacteria isolated from urban wastewaters. Res. Microbiol. 163, 92–100. doi:10.1016/j.resmic.2011.10.010

Moura, A., Soares, M., Pereira, C., Leitão, N., Henriques, I., Correia, A., 2009. INTEGRALL: A database and search engine for integrons, integrases and gene cassettes. Bioinformatics 25, 1096–1098. doi:10.1093/bioinformatics/btp105

Nataro, J.P., Kaper, B.J., 1998. Diarrheagenic *Escherichia coli* strains. Clin. Microbiol. Rev. 11, 147–201.

National Center for Emerging and Zoonotic Infectious Diseases, 2011. CDC Estimates of Foodborne Illness in the United States, Centers for Disease Control and Prevention. doi:10.1111/j.1753-4887.2010.00286.x

Osterblad, M., Pensala, O., Peterzéns, M., Heleniusc, H., Huovinen, P., 1999. Antimicrobial susceptibility of *Enterobacteriaceae* isolated from vegetables. J. Antimicrob. Chemother. 43, 503–9. doi:10.1093/jac/43.4.503

Pannaraj, P.S., Bard, J.D., Cerini, C., Weissman, S.J., 2015. Pediatric carbapenem-resistant *Enterobacteriaceae* in Los Angeles, California, a high-prevalence region in the United States. Pediatr. Infect. Dis. J. 34, 11–6. doi:10.1097/INF.0000000000000471

Paton, A., Paton, J., 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex assays for *stx1*, *stx2*, *eaeA*, enterohemorrhagic *E. coli hlyA, rfbO111,* and *rfbO157*. J. Clin. Microbiol. 36, 598–602.

Pereira, A., Santos, A., Tacão, M., Alves, A., Henriques, I., Correia, A., 2013. Genetic diversity and antimicrobial resistance of Escherichia coli from Tagus estuary (Portugal). Sci. Total Environ. 461–462, 65–71. doi:10.1016/j.scitotenv.2013.04.067

Pomerleau, J., Lock, K., McKee, M., 2006. The burden of cardiovascular disease and cancer attributable to low fruit and vegetable intake in the European Union: differences between old and new Member States. Public Health Nutr.

9, 575–583. doi:10.1079/PHN2005910

Potron, A., Poirel, L., Rondinaud, E., Nordmann, P., 2013. Intercontinental spread of OXA-48 beta-lactamase-producing *Enterobacteriaceae* over a 11-year period, 2001 to 2011. Eurosurveillance 18. doi:10.2807/1560-7917.ES2013.18.31.20549

Richardson, H.Y., Nichols, G., Lane, C., Lake, I.R., Hunter, P.R., 2009. Microbiological surveillance of private water supplies in England: the impact of environmental and climate factors on water quality. Water Res. 43, 2159–2168. doi:10.1016/j.watres.2009.02.035

Rodrigues, C., Machado, E., Pires, J., Ramos, H., Novais, Â., Peixe, L., 2015. Increase of widespread A, B1 and D *Escherichia coli* clones producing a high diversity of CTX-M-types in a Portuguese hospital. Future Microbiol. 10, 1125–1131. doi:10.2217/fmb.15.38

Schwaiger, K., Helmke, K., Hölzel, C.S., Bauer, J., 2011. Antibiotic resistance in bacteria isolated from vegetables with regards to the marketing stage (farm vs. supermarket). Int. J. Food Microbiol. 148, 191–196. doi:10.1016/j.ijfoodmicro.2011.06.001

Shahada, F., Chuma, T., Kosugi, G., Kusumoto, M., Iwata, T., Akiba, M., 2013. Distribution of extended-spectrum cephalosporin resistance determinants in *Salmonella enterica* and *Escherichia coli* isolated from broilers in southern Japan. Poult. Sci. 92, 1641–9. doi:10.3382/ps.2012-02934

Söderström, A., Osterberg, P., Lindqvist, A., Jönsson, B., Lindberg, A., Blide Ulander, S., Welinder-Olsson, C., Löfdahl, S., Kaijser, B., De Jong, B., Kühlmann-Berenzon, S., Boqvist, S., Eriksson, E., Szanto, E., Andersson, S., Allestam, G., Hedenström, I., Ledet Muller, L., Andersson, Y., 2008. A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. Foodborne Pathog. Dis. 5, 339–49. doi:10.1089/fpd.2007.0065

Szmolka, A., Nagy, B., 2013. Multidrug resistant commensal *Escherichia coli* in animals and its impact for public health. Front. Microbiol. 4, 1–13. doi:10.3389/fmicb.2013.00258

Tacão, M., Correia, A., Henriques, I., 2012. Resistance to broad-spectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of $bla_{CTX\text{-}M}$-like genes. Appl. Environ. Microbiol. 78, 4134–4140. doi:10.1128/AEM.00359-12

Tacão, M., Correia, A.C.M., Henriques, I.S., 2015. Low prevalence of carbapenem-resistant bacteria in river water: resistance is mostly related to intrinsic mechanisms. Microb. Drug Resist. 21, 497–506. doi:10.1089/mdr.2015.0072

Tacão, M., Moura, A., Correia, A., Henriques, I., 2014. Co-resistance to different classes of antibiotics among ESBL-producers from aquatic systems. Water Res. 48, 100–107. doi:10.1016/j.watres.2013.09.021

Valverde, A., Cantón, R., Garcillán-Barcia, M.P., Novais, Â., Galán, J.C., Alvarado, A., De La Cruz, F., Baquero, F., Coque, T.M., 2009. Spread of $bla_{CTX\text{-}M\text{-}14}$ is driven mainly by IncK plasmids disseminated among *Escherichia coli* phylogroups A, B1, and D in Spain. Antimicrob. Agents Chemother. 53, 5204–5212. doi:10.1128/AAC.01706-08

Versalovic, J., Koeuth, T., Lupski, J.R., 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19, 6823–31. doi:10.1093/nar/19.24.6823

Viswanathan, P., Kaur, R., 2001. Prevalence and growth of pathogens on salad vegetables, fruits and sprouts. Int. J. Hyg. Environ. Health 203, 205–213. doi:10.1078/S1438-4639(04)70030-9

von Wintersdorff, C.J.H., Penders, J., van Niekerk, J.M., Mills, N.D., Majumder, S., van Alphen, L.B., Savelkoul, P.H.M., Wolffs, P.F.G., 2016. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Front. Microbiol. 7, 173. doi:10.3389/fmicb.2016.00173

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L.H., Karch, H., Reeves, P.R., Maiden, M.C.J., Ochman, H., Achtman, M., 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol. Microbiol. 60, 1136–51. doi:10.1111/j.1365-2958.2006.05172.x

Zurfluh, K., Nüesch-Inderbinen, M., Morach, M., Zihler Berner, A., Hächler, H., Stephan, R., 2015. Extended-spectrum-β-lactamase-producing *Enterobacteriaceae* isolated from vegetables imported from the Dominican Republic, India, Thailand, and Vietnam. Appl. Environ. Microbiol. 81, 3115–3120. doi:10.1128/AEM.00258-15

CHAPTER 4: *Genome analysis of two multidrug-resistant Escherichia coli O8:H9-ST48 strains isolated from lettuce*

Gene, 2021
doi: 10.1016/j.gene.2021.145603

1. ABSTRACT

Vegetables may become contaminated with antibiotic-resistant bacteria from farm-to-fork. Here we report draft genome sequences of two multidrug-resistant *Escherichia coli* isolated from lettuce. Whole genomes of strains Y15 V.22 and Y15 V.54 were sequenced. Available tools were used to inspect for virulence factors (VF), metals tolerance, resistome and mobilome features. The predicted genome sizes were 5,4 Mb and 6,2 Mb for Y15 V.22 and Y15 V.54, respectively, both with 50.7% GC content, ST48 and serotype O8:H9. Resistome analysis showed genes encoding resistance to β-lactams, sulphonamides, trimethoprim, tetracyclines and macrolides. Cobalt, cadmium, zinc and copper tolerance determinants were identified in both. VF detected included genetic determinants related to toxin production, adherence and invasion. SNPs and VF content analysis showed a close relatedness to ETEC. Putative genomic islands, prophage and CRISPR sequences were predicted. The genome sequences here reported will aid in understanding antibiotic resistance transfer between vegetables consumed raw and humans.

Keywords:

Escherichia coli; Multidrug-resistance; Food contamination; Antibiotic resistance.

2. INTRODUCTION

The environment is important for the evolution and spread of antibiotic resistance (Surette and Wright, 2017). The transfer of resistance from the environment to humans and other animals is still poorly understood. Yet, contaminated food has been suggested as a key vehicle (Araújo et al., 2017; Liu et al., 2017; Luo et al., 2017). Despite remarkable advances in food technology, foodborne illnesses are a main cause of morbidity and preventable death worldwide (Jones et al., 2008). Vegetables have been identified as the source of several foodborne outbreaks caused by multidrug-resistant strains (Araújo et al., 2017; Liu et al., 2017). For instance, in the last decades, disease outbreaks caused by pathogens associated with leafy green vegetables consumption have been increasingly reported on a global scale (Jones et al., 2008). Although a relationship has been found between this increase and greater intake of fresh raw vegetables (Mercanoglu Taban and Halkman, 2011), outbreaks have increased beyond what can be explained by raise in consumption. In Europe some important outbreaks of food poisoning associated with consumption of fresh vegetables were linked to intake of lettuce contaminated with enterotoxigenic *Escherichia coli* ETEC (Ethelberg et al., 2010) or Shiga-toxin-producing *Escherichia coli* STEC O157 (Friesema et al., 2008).

Thus, the risk to human health associated with pathogens in fresh vegetables clearly cannot be assessed by simply detecting a genus or species in a culture plate. Molecular approaches targeting virulence and antibiotic resistance genes have a great potential for improving the specificity and risk predictive value of microbial assessment for public health purposes.

In a previous work, several multidrug-resistant *E. coli* strains were detected which were retrieved from vegetables and irrigation water in small domestic farms, in a geographic zone where contamination of aquatic systems with antibiotic resistant bacteria has been reported (Araújo et al., 2017). In this way, our goal was to further assess the risk associated to the presence of these multidrug-resistant *E. coli* isolated from lettuce, that is usually eaten raw, by analysing their resistome, virulence factors and mobilization platforms, through whole-genome sequence analysis.

3. MATERIALS AND METHODS

3.1. Bacterial strains and DNA purification

From a previous study (Araújo et al., 2017), two *E. coli* isolates (Y15 V.22 and Y15 V.54) were selected for whole-genome sequence analysis. Both isolates were recovered from lettuce and displayed distinct rep-PCR profiles. Y15 V.22 shared identical rep-PCR profile with isolates recovered from irrigation water (Araújo et al. 2017), suggesting the water as the origin of contamination.

Y15 V.22 and Y15 V.54 showed multidrug resistance traits, including resistance to streptomycin, tetracycline and the combination sulfamethoxazole/trimethoprim. Both affiliated to Clermont phylogroup A (Araújo et al. 2017).

Genomic DNA of Y15 V.22 and Y15 V.54 was purified using the Wizard Genomic DNA Purification kit following the manufacturer's protocol (Promega).

3.2. Whole genome sequencing and draft genome analysis

Whole-genomes were sequenced using Ion Proton System (ThermoFisher Scientific) with the Ion PI chip that generates reads with up to 200 bp in size. The assembly was carried out with SPAdes v.3.13.0 and annotation was performed using the Rapid Annotation using Subsystems Technology (RAST) server (http://rast.nmpdr.org). Draft genomes were analysed with tools available at the Center for Genomic Epidemiology (CGE; http://www.genomicepidemiology.org/), to investigate the presence of resistance genes (ResFinder 3.2) and plasmids (PlasmidFinder 2.0). Additionally, the sequence type (MLST 2.0 #1), serotype (SerotypeFinder 2.0), and FimH and FumC types (CHTyper 1.0) were determined at CGE site. The resistome was further predicted using the resistance gene identifier tool available at the Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca/), and virulence factors were predicted with VFanalyzer at Virulence factors database (VFDB; http://www.mgc.ac.cn/VFs/).

These genomes were inspected for metal resistance genes by analysing the RAST subsystem related to virulence, disease and defence – resistance to antibiotics and toxic compounds. Prophage sequences and clustered regularly interspaced short palindromic repeats (CRISPR) were analyzed using PHAge Search Tool Enhanced Release (PHASTER) (Arndt et al., 2016) and CRISPRCasFinder (Couvin et al., 2018), respectively.

Genomic islands were predicted and analyzed using the Genomic Island Prediction Software (GIPSy) v.1.1.2 (Soares et al., 2016), using *E. coli* K-12 MG1655 genome as reference. The program BRIG v.0.95 (BLAST Ring Image Generator) (Alikhan et al., 2011) was used to visualize the genomic similarity between *E. coli* genomes, highlighting putative genomic islands and prophage sequences.

The Reference sequence Alignment-based Phylogeny builder (REALPHY 1.12; https://realphy.unibas.ch/realphy/; Bertels et al., 2014), was used to infer phylogenetic trees from whole genome sequence data available from pathogenic *E.coli* strains (intestinal and extraintestinal pathogenic *E. coli*), using default parameters and performing 2 separate runs using each genome as a single reference sequence. The virulence factors content of pathogenic *E.coli* strains were also compared with *E. coli* Y15 V.22 and Y15 V.54 by performing a cluster analysis with PRIMER v6 software using UPGMA method (group average method) applying Simple matching correlation analysis (Clarke and Gorley, 2006).

3.3. Nucleotide sequence accession number

Draft genomes of Y15 V.22 and Y15 V.54 were deposited in GenBank/NCBI database under the accession numbers WTST00000000 and WTSU00000000, respectively. The accession numbers of all genome sequences used in this work are listed in supplemental material, Table S1.

4. RESULTS AND DISCUSSION

For Y15 V.22 the predicted draft genome size was 5,429,619 bp, organized in 125 contigs, with a N50 of 93,772 and 6,442 predicted coding sequences. For Y15 V.54 the draft genome consisted of 6,166,249 bp arranged in 107 contigs, with a N50 of 106,069 and 6,928 predicted coding sequences. For both, the estimated GC content was 50.7%. Both isolates belonged to ST48, serotype O8:H9 and fimH54-C11.

In both, genes encoding resistance to different antibiotics were detected: beta-lactams (*ampC1*), aminoglycosides (*aadA5*), trimethoprim (*dfrA17*), sulphonamides (*sul2*), tetracyclines (*tetA*) and macrolides (*mphA*). The genes encoding *aadA5*, *dfA17* and *sul2* were identified in a class 1 integron array (Figure 1). This array showed 99.7%-99.8% similarity to that identified in the pTB402 plasmid characterized in a *E. coli* strain isolated from feces in China (accession no. CP034786). An hybrid PcH1 promoter (TGGACA – TAAACT) was positioned within *intI*1 followed by a P2 promoter (TTGTTA – TACAGT).

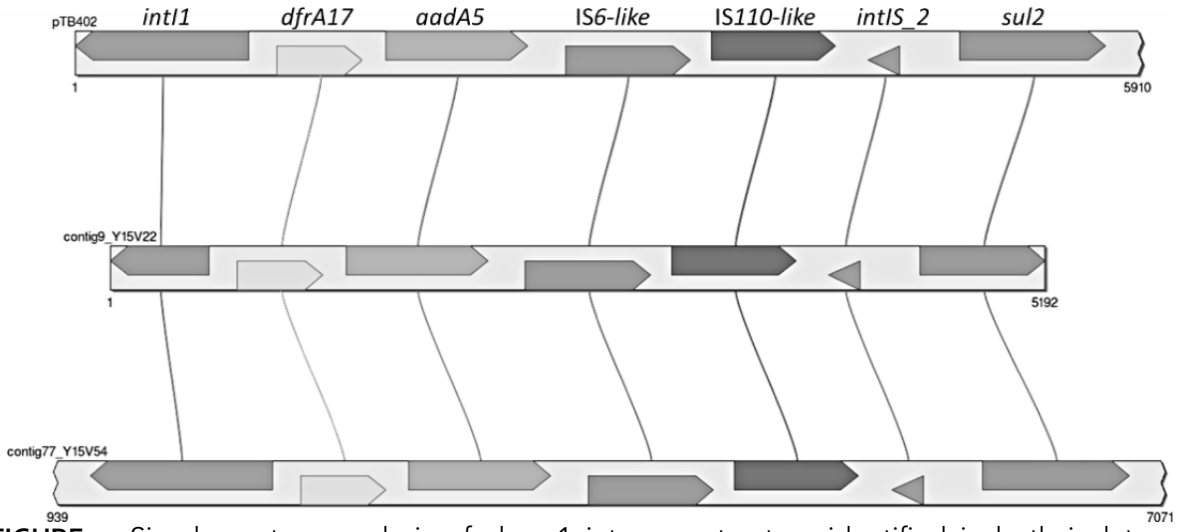


FIGURE 1. Simple synteny analysis of class 1 integron structure identified in both isolates (Y15 V.22 and Y15 V.54) and in plasmid pTB402 of *E. coli* ECZP248 (GenBank accession no. CP034786). Lines indicate conserved genes.

Co-selection of antibiotic and metal resistance has been reported previously (Henriques et al., 2016), suggesting metals as selectors of antibiotic resistance, e.g. in agriculture production. Results showed that genes related to cobalt, cadmium, zinc and copper tolerance were present in the genomes of both

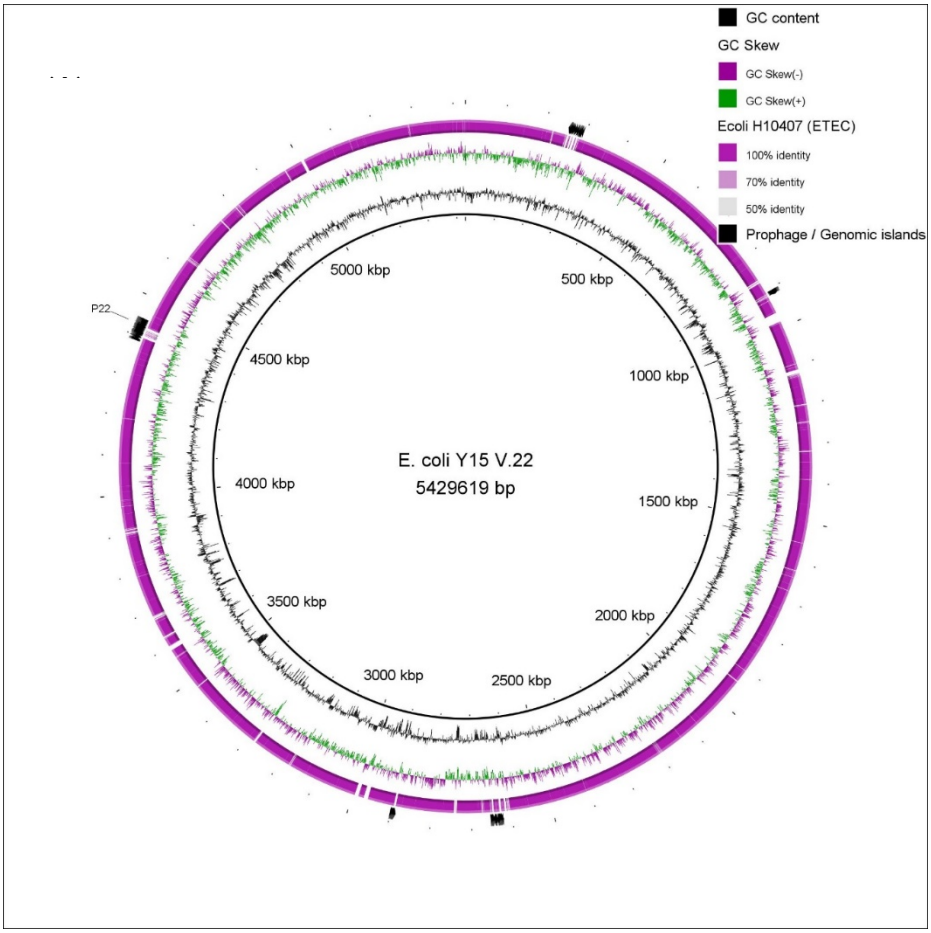
isolates. To highpoint, these *E. coli* strains were both isolated from lettuce irrigated with water from household wells in a geographic area with high industrial activity, where contamination with metals has been reported including in water (Araújo et al., 2017; Cabral-Pinto et al., 2020).

Plasmid finder identified a pO111-like plasmid replicon with 98.64% similarity in both genomes. No metal resistance genes or virulence factors were associated to this replicon. In Y15 V.54, the contig corresponding to the replicon contained an *ampC*-like gene, encoding resistance to beta-lactams.

In both isolates, additional evidences of mobilization elements were detected, namely IS-like transposase elements belonging to the IS*26* and IS*5075* families (IS*6*- and IS*110*-like elements, respectively), and also a phage integrase-encoding gene, *intS_2*. The Y15 V.22 and Y15 V.54 genomes presented 9 and 13 CRISPR, respectively, with evidence levels from 1 to 4. The predicted number of genomic islands in Y15 V.22 and Y15 V.54 genomes was 4 and 23, respectively (Figure 2), varying in sizes from 8.9 Kb to 36.9 Kb in Y15 V.22, and 5.2 Kb to 37.4 Kb in in Y15 V.54. The difference in genome size between these two genomes, ~700 Kb can be attributed to the higher number of putative genomic islands predicted in Y15 V.54, accounting for 332.6 Kb. In the putative genomic islands identified in Y15 V.22 genome it was possible to identify CDS mostly related to putative mobile elements and type I fimbriae. Likewise, putative genomic islands predicted for Y15 V.54, presented CDS related to mobile elements (e.g insertion sequences, transposases), but also encoding phage-related proteins, or linked to Type IV secretion or multidrug efflux systems. In both, intact prophage sequences were identified (Figure 2).

As recently reviewed by Desvaux and co-authors (Desvaux et al., 2020), genomic islands are widespread in *E. coli* and are important players in genome plasticity, thus supporting their rapid adaptation. Even though the occurrence of key genomic islands, particularly pathogenicity islands, has been observed mostly in pathogenic strains, some have been identified on both commensal and pathogenic *E.coli* strains, highlighting the adjustable behaviour of *E. coli* (Desvaux et al., 2020).

Virulence factors were predicted in both genomes, namely related to adherence (*elfC*, *elfG, hcpB, hcpC, fimD, fimH*), invasion (*ibeB*, *ibeC*), toxins



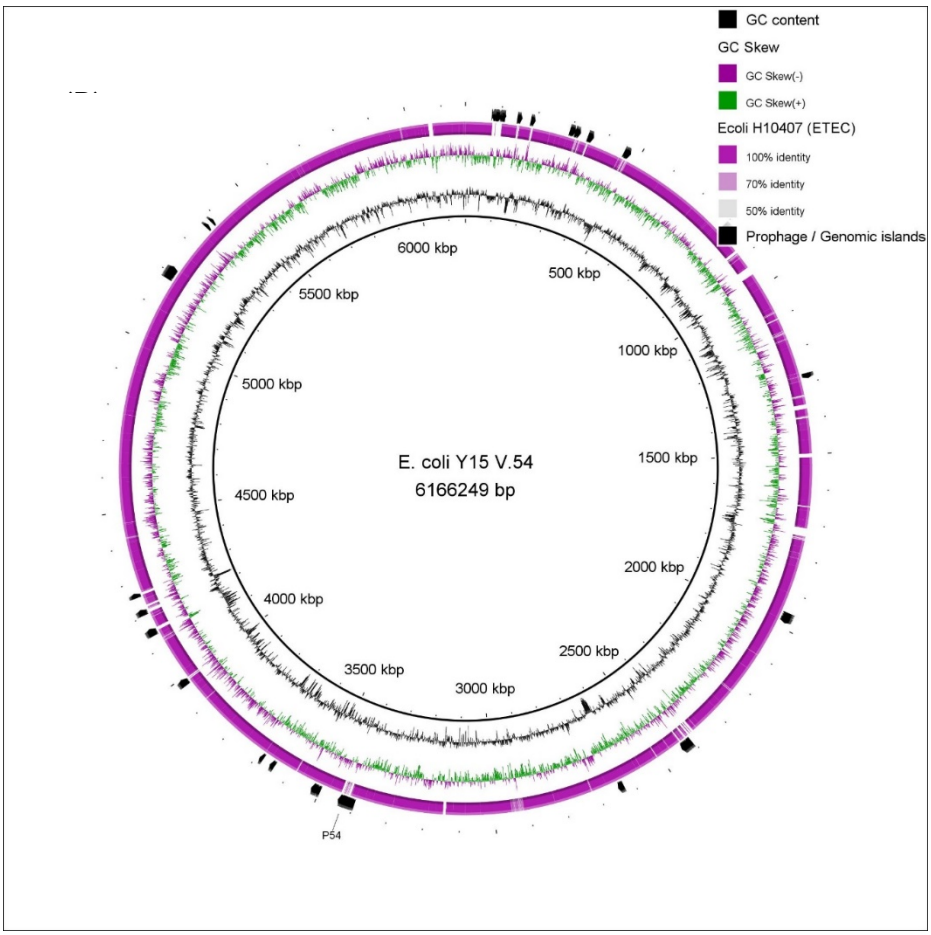


FIGURE 2. Circular genome comparison of ETEC *E. coli* H10407 and both *E. coli* isolated from lettuce: (A) Y15 V.22 and (B) Y15 V.54. Putative genomic islands and prophage sequences are presented in black. BRIG performed the alignment using a local BLAST + with the standard parameters (50% lower–70% upper cut-off for identity and E-value of 10). The ring colour gradients correspond to varying degrees of identity of BLAST matches. Circular genomic maps also include information on GC Skew and GC content.

(*hlyE/clyA*), and metabolic adaptation (*gad*). Some virulence factors were unique in the genomes, as for example virulence factors related to secretion system (*aec15*) and adherence (*elfA, hcpA* and *fimA, fimC fimF, fimG fimI*), identified only in Y15 V.54 genome (Table S2). To note that the virulence genes *hcpA-hcpC* related to the haemorrhagic *E. coli* pilus have been identified also in *E. coli* pathogenic strains, including *E. coli* uropathogenic (UPEC), enterohaemorragic (EHEC), enterotoxic (ETEC) and enteroaggregative (EAEC). Furthermore, the presence of genetic determinants encoding the hemolysin ClyA, detected in both genomes, has been associated to ETEC pathogenesis (Del Canto et al., 2011).

Cluster analysis based on a presence/absence matrix that included virulence factors in these genomes together with representatives of *E. coli* pathotypes available at VFDB is shown in Figure 3. Though presenting an atypical ETEC virulome profile, e.g. the absence of genes encoding heat-labile and/or heat-stable enterotoxins, cluster analysis indicates that Y15 V.22 and Y15 V.54 group with ETEC strains, sharing mostly virulence factors related to adherence, invasion and toxins (Table S2).

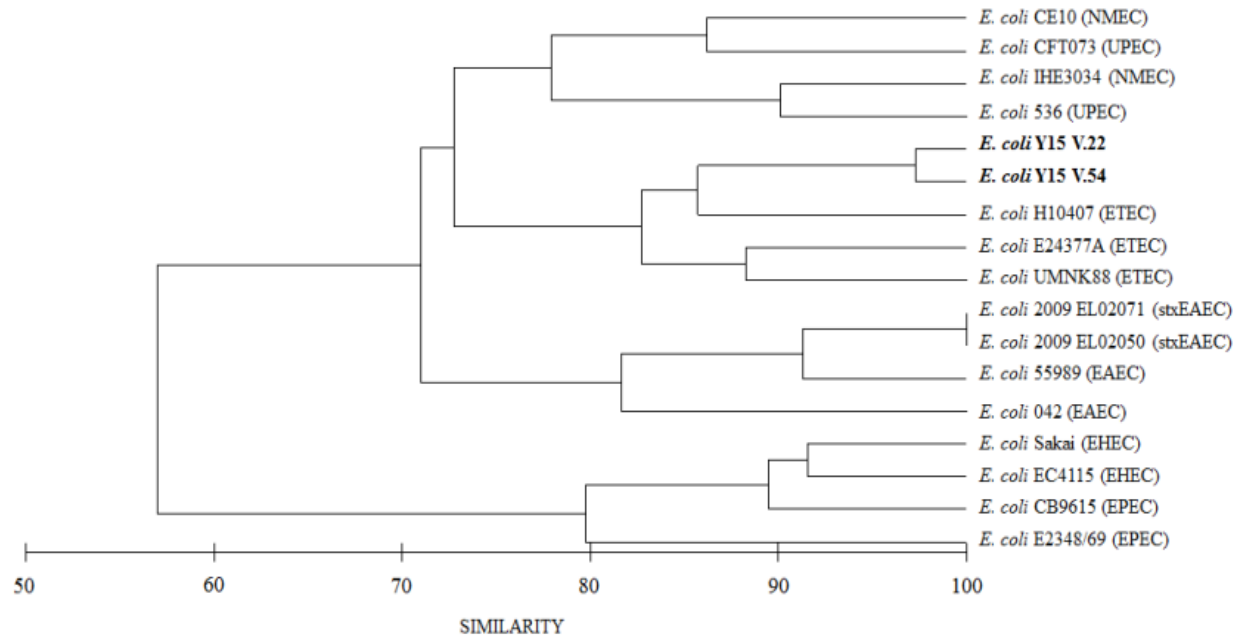


FIGURE 3. Cluster analysis of virulence factors predicted against VFDB based on whole-genome analysis of *E. coli* strains isolated from lettuce (Y15 V.22 and Y15 V.54) and representatives of *E. coli* pathotypes: uropathogenic *E. coli* (UPEC), Neonatal meningitis-associated *E. coli* (NMEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC) and shiga toxin-producing

enteroaggregative *E. coli* (stxEAEC). Using simple matching coefficient and unweighted pair group method using arithmetic averages cluster methods.

Whole-genome based analysis performed with REALPHY also showed a close relationship with ETEC strains, further suggesting a phylogenetic proximity with this pathotype (Figure 4). Moreover, our isolates affiliated with the same ST as the ETEC strain H10407-ST48. When compared with *E. coli* H10407 ETEC strain (Figure 2) it is possible to observe a high similarity between sequences but with unique putative genomic islands identified in both genomes, as well as prophage sequences.

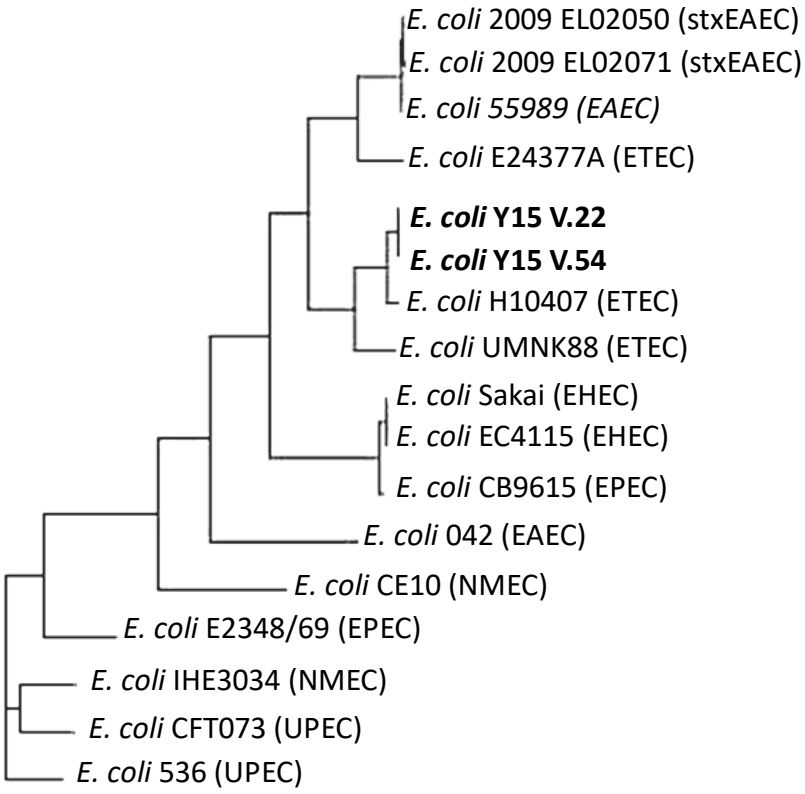


FIGURE 4. Phylogenetic analysis based on whole-genome-sequences of *E. coli* isolated from lettuce (in bold) and selected representatives of pathogenic *E. coli* strains. Phylogenetic tree analysis was performed using REALPHY, applying the PhyML algorithm for tree constructing (merged tree with Y15 V.22 and Y15 V.54 as reference genomes).

E. coli ST48 has been isolated previously worldwide and from different environmental and clinical sources (Enterobase, https://enterobase.warwick.ac.uk; PATRIC, https://www.patricbrc.org/), suggesting its ability to colonize different hosts and settings.

As far as we know this is the first report of an MDR *E. coli* O8:H9-ST48 in Portugal, closely related to ETEC strains. *E. coli* ST48 has been associated with bla_{CTX-M}-carrying plasmids, but also bla_{NDM} and *mcr-1* in humans and food sources (Liu et al., 2017; Luo et al., 2017), pointing out to a possible role of this clone in spreading antibiotic resistance between environmental and human settings.

5. CONCLUSIONS

Consumption of raw vegetables are undoubtedly beneficial to human health. Nevertheless, contaminated vegetables may pose risks to humans related to contamination with antibiotic-resistant bacteria. In fact, this is one of the most obvious routes of antibiotic resistance spread between the environment and humans. This study provides the analysis of the genomes of potentially pathogenic MDR *E. coli*, representing an added value for understanding this phenomenon. The strains analyzed in this study were isolated from lettuce with identical typing clones recovered from water, suggesting irrigation water as the most likely source of vegetable contamination. While the upstream origin is unknown, the existence of several livestock farms in the vicinity of the wells indicates an animal origin for these strains. The results presented here emphasize the importance of applying proper food production. Primarily, it is urgent to implement routine microbiological monitoring of the final product, but also focus on potential contamination points in the field. Produce should be cold stored and washed carefully with chlorinated water, in household. This study also highlights the need to implement antibiotic resistance surveillance programs in food-derived bacterial isolates.

6. ACKNOWLEDGMENTS

This work was funded by the international cooperation project "ARTEMan: Antibiotic resistance transfer between environmental and human settings." financed by the Brazilian agency Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Portuguese agency Fundação para a Ciência e Tecnologia (FCT). Authors acknowledge FCT financing to Marta Tacão (CEEC/01304/2017) and Susana Araújo (SFRH/BD/52573/2014). Thanks are due for the financial support to CESAM (UIDP/50017/2020+UIDB/50017/2020), through national funds.

7. REFERENCES

Alikhan, N.F., Petty, N.K., Ben Zakour, N.L., Beatson, S.A., 2011. BLAST Ring Image Generator (BRIG): Simple prokaryote genome comparisons. BMC Genomics 12, 402. doi:10.1186/1471-2164-12-402

Araújo, S., A.T. Silva, I., Tacão, M., Patinha, C., Alves, A., Henriques, I., 2017. Characterization of antibiotic resistant and pathogenic *Escherichia coli* in irrigation water and vegetables in household farms. Int. J. Food Microbiol. 257, 192–200. doi:10.1016/j.ijfoodmicro.2017.06.020

Arndt, D., Grant, J.R., Marcu, A., Sajed, T., Pon, A., Liang, Y., Wishart, D.S., 2016. PHASTER: a better, faster version of the PHAST phage search tool. Nucleic Acids Res. doi:10.1093/nar/gkw387

Bertels, F., Silander, O.K., Pachkov, M., Rainey, P.B., Van Nimwegen, E., 2014. Automated reconstruction of whole-genome phylogenies from short-sequence reads. Mol. Biol. Evol. doi:10.1093/molbev/msu088

Cabral-Pinto, M.M.S., Inácio, M., Neves, O., Almeida, A.A., Pinto, E., Oliveiros, B., Ferreira da Silva, E.A., 2020. Human health risk assessment due to agricultural activities and crop consumption in the surroundings of an industrial area. Expo. Heal. 12, 629–640. doi:10.1007/s12403-019-00323-x

Clarke, K.R., Gorley, R.N., 2006. PRIMER v6: User Manual/Tutorial Plymouth Routines In Multivariate Ecological Research. PRIMER-E.

Couvin, D., Bernheim, A., Toffano-Nioche, C., Touchon, M., Michalik, J., Néron, B., Rocha, E.P.C., Vergnaud, G., Gautheret, D., Pourcel, C., 2018. CRISPRCasFinder, an update of CRISRFinder, includes a portable version,

enhanced performance and integrates search for Cas proteins. Nucleic Acids Res. doi:10.1093/nar/gky425

Del Canto, F., Valenzuela, P., Cantero, L., Bronstein, J., Blanco, J.E., Blanco, J., Prado, V., Levine, M., Nataro, J., Sommerfelt, H., Vidal, R., 2011. Distribution of classical and nonclassical virulence genes in enterotoxigenic *Escherichia coli* isolates from Chilean children and tRNA gene screening for putative insertion sites for genomic islands. J. Clin. Microbiol. doi:10.1128/JCM.02473-10

Desvaux, M., Dalmasso, G., Beyrouthy, R., Barnich, N., Delmas, J., Bonnet, R., 2020. Pathogenicity factors of genomic islands in intestinal and extraintestinal *Escherichia coli*. Front. Microbiol. 11, 2065. doi:10.3389/fmicb.2020.02065

Ethelberg, S., Lisby, M., Böttiger, B., Schultz, A.C., Villif, A., Jensen, T., Olsen, K.E., Scheutz, F., Kjelsø, C., Müller, L., 2010. Outbreaks of gastroenteritis linked to lettuce, Denmark, January 2010. Eurosurveillance. doi:10.2807/ese.15.06.19484-en

Friesema, I., Sigmundsdottir, G., van der Zwaluw, K., Heuvelink, A., Schimmer, B., de Jager, C., Rump, B., Briem, H., Hardardottir, H., Atladottir, A., Gudmundsdottir, E., van Pelt, W., 2008. An international outbreak of Shiga toxin-producing *Escherichia coli* O157 infection due to lettuce, September - October 2007. Eurosurveillance 13, 1–5.

Henriques, I., Tacão, M., Leite, L., Fidalgo, C., Araújo, S., Oliveira, C., Alves, A., 2016. Co-selection of antibiotic and metal(loid) resistance in Gram-negative epiphytic bacteria from contaminated salt marshes. Mar. Pollut. Bull. 109, 427–434. doi:10.1016/j.marpolbul.2016.05.031

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., Daszak, P., 2008. Global trends in emerging infectious diseases. Nature. doi:10.1038/nature06536

Liu, Z., Wang, Y., Walsh, T.R., Liu, D., Shen, Z., Zhang, R., Yin, W., Yao, H., Li, J., Shen, J., 2017. Plasmid-mediated novel *bla*$_{NDM-17}$ gene encoding a carbapenemase with enhanced activity in a sequence type 48 *Escherichia coli* strain. Antimicrob. Agents Chemother. 61. doi:10.1128/AAC.02233-16

Luo, J., Yao, X., Lv, L., Doi, Y., Huang, X., Huang, S., Liu, J.H., 2017. Emergence of *mcr-1* in *Raoultella ornithinolytica* and *Escherichia coli* isolates from retail vegetables in China. Antimicrob. Agents Chemother. 61. doi:10.1128/AAC.01139-17

Mercanoglu Taban, B., Halkman, A.K., 2011. Do leafy green vegetables and their ready-to-eat [RTE] salads carry a risk of foodborne pathogens? Anaerobe 17,

286–287. doi:10.1016/j.anaerobe.2011.04.004

Soares, S.C., Geyik, H., Ramos, R.T.J., de Sá, P.H.C.G., Barbosa, E.G.V., Baumbach, J., Figueiredo, H.C.P., Miyoshi, A., Tauch, A., Silva, A., Azevedo, V., 2016. GIPSy: Genomic island prediction software. J. Biotechnol. doi:10.1016/j.jbiotec.2015.09.008

Surette, M.D., Wright, G.D., 2017. Lessons from the Environmental Antibiotic Resistome. Annu. Rev. Microbiol. 71, 309–329. doi:10.1146/annurev-micro-090816-093420

CHAPTER 5: *Carbapenem-resistant bacteria over a wastewater treatment process: carbapenem-resistant Enterobacteriaceae in untreated wastewater and intrinsically-resistant bacteria in final effluent*

Science of the Total Environment, 2021
doi: 10.1016/j.scitotenv.2021.146892

1. ABSTRACT

Although urban wastewater treatment plants (UWWTPs) are important sites for antibiotic resistance eimination, their limitation in producing resistance-free effluents is recognized. Despite the critical importance of carbapenems to human health, the fate and diversity of carbapenem-resistant bacteria (CRB) in UWWTPs is still poorly understood. We analysed CRB from different treatment stages in a UWWTP applying UV-C radiation. After secondary treatment, bacteria grown in mFC medium reduced in 1.9-log units ($p<0.0001$), while CRB counts reduction (0.2-log units) was not significant. UV-C reduced the abundance of total bacteria and CRB (1.8 and 2.4-log units, respectively, relative to values after secondary treatment). Yet after incubation in the dark, CRB increased (0.6-log units) in UV-treated samples. Albeit in low amounts, carbapenem-resistant *Enterobacteriaceae* (CRE) were detected in raw wastewater being absent from the final effluent. Thirty-four CRE isolates were identified as *Citrobacter*, *Enterobacter*, *Leclercia* and *Lelliottia*. These were multiresistant and yielded classes 1, 2 and 3 integrons (94%, 88% and 88%, respectively). In all CRE isolates, *bla*$_{GES-5}$ was found in the integrons. Three isolates were selected for whole-genome sequenicing; in *Citrobacter braaki* (n=2) *bla*$_{GES-5}$ was part of class 3 integrons, while in *Lelliotia* sp. RWM.1 *bla*$_{GES-5}$ was in a class 1 integron with a novel cassette array (*bla*$_{OXA-10}$/*aacA4*-*bla*$_{GES-5}$-*bla*$_{BEI-1}$). These integrons were in contigs with high similarity with mobilizable plasmids. Genes encoding resistance to other antibiotics were detected in these isolates. In the final effluent, CRB were predominantly affiliated with *Stenotrophomonas maltophilia*. UV-C radiation significantly reduced the abundance and prevalence of CRB. Bacteria intrinsically-resistant to carbapenems were cultivated after all treatment stages, while CRE only in raw wastewater. In these samples, we detected CRE with *bla*$_{GES-5}$, in integrons and plasmids. This raises concern as horizontal gene transfer may occur within these systems. Carbapenem resistance surveillance in UWWTPs is essential to implement mitigation measures in a timely manner.

Keywords: Antibiotic resistance; Carbapenems; UWWTPs; *Enterobacteriaceae*; *bla*$_{GES-5}$.

2. INTRODUCTION

Carbapenems are used in human medicine, frequently as the last therapeutic option to combat serious infections caused by Gram-negative bacteria. In the last decades, emergence and dissemination of carbapenem resistance have been witnessed throughout the world (Potter et al., 2016), posing a serious threat to public health due to the significant limitations that we face in terms of antibiotic therapy.

Urban wastewater treatment plants (UWWTPs) have been suggested as important sources of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) (Manaia et al., 2016; Michael et al., 2013; Rizzo et al., 2013), and potential niches for ARGs transfer (Moura et al., 2010; Rizzo et al., 2013; Schlüter et al., 2007). Typically, urban wastewater plants' treatments rely on the removal of solids and on biological processes which were primarily designed for organic matter, nutrients and other contaminants removal (Pruden, 2014). Disinfection with UV-C radiation has been used to reduce the microbial load of treated wastewater, and therefore is considered a promising approach to attenuate the antibiotic resistance burden (Fatta-kassinos et al., 2015; Guo et al., 2013; Hu et al., 2016) without the formation of toxic by-products (Pei et al., 2019). However, the efficacy of UV-C (wavelenghts of 200–260 nm) radiation in removing ARB and ARGs is influenced by different factors and frequently leads to low removal rates of these biological contaminants (Guardabassi et al., 2002; Hu et al., 2016; McKinney and Pruden, 2012; Munir et al., 2011; Silva et al., 2018).

The presence of carbapenem-resistant bacteria (CRB) has been reported in treated and untreated wastewater (Hrenovic et al., 2017a; Serna-Galvis et al., 2018; Zhang et al., 2020). However, the number of reported studies is still scarce and in most cases these studies focused on the quantification of ARB and ARGs, providing little information on CRB phylogeny and genomic characteristics. This information is essential to better assess the risk of CRB spread from UWWTPs to the environment. In a general sense, human-related CRB with acquired resistance mechanisms represent a higher risk than environmental species with intrinsic resistance to carbapenems. Among the latter, *Stenotrophomonas maltophilia* has been reported in high abundance in untreated and treated wastewater (Hrenovic et al., 2017a; Kim et al., 2018), along with sporadic reports of other environmental

CRB belonging to genera such as *Aeromonas*, *Chryseobacterium*, *Cupriavidus* and *Ralstonia* (Reinke et al., 2020; Zhang et al., 2020).

Regarding human-related CRB, the presence of carbapenem-resistant *Enterobacteriaceae* (CRE) has been reported in hospital and municipal raw wastewater (Chagas et al., 2011; Manageiro et al., 2014b; Serna-Galvis et al., 2018). In a 2013 report by US Centers for Disease Control and Prevention (CDC), CRE were listed as one of the three most urgent antibiotic resistance threats (Centers for Disease Control and Prevention, 2013). Likewise, in 2017 the World Health Organization (WHO) listed CRE as critical priority pathogens to be targeted for research and development (WHO, 2017). In Europe, healthcare-associated CRE have been increasingly reported in the last decade (Magiorakos et al., 2017) but environmental and community reports are still infrequent (Harmon et al., 2019; Kelly et al., 2017; Tacão et al., 2015; Teixeira et al., 2020).

The production of carbapenemases is the most common and efficient carbapenem resistance mechanism among CRE. Carbapenemases are a diverse group of enzymes belonging to Ambler classes A, B and D (Iovleva and Doi, 2017; Queenan and Bush, 2007; Tzouvelekis et al., 2012). Class A carbapenemases include the most clinically relevant and widely disseminated KPC (*Klebsiella pneumoniae* carbapenemases) and also less frequent enzymes such as SME, NMC/IMI, SFC-1, SHV-38 and a few variants of GES (Guiana extended-spectrum β-lactamase) (Bush, 2018b; Henriques et al., 2004; Nordmann and Poirel, 2014; Poirel et al., 2003a). GES enzymes are capable of hydrolysing broad-spectrum cephalosporins, whereas in some variants (e.g. GES-2, GES-4, GES-5, GES-6, GES-14, and GES-18) the substitution of a glycine for either an asparagine or a serine at Ambler's position 170 resulted in an extended spectrum of activity against carbapenems (Barrios et al., 2012; Naas et al., 2008; Nordmann and Poirel, 2014). *bla*$_{GES}$ genes have been found in association with mobilizable and conjugative plasmids (Poirel et al., 2010a) and are detected commonly as gene cassettes in class 1 or class 3 integrons (Correia et al., 2003; Poirel et al., 2010a; Teixeira et al., 2020; Xu et al., 2018). Their mobilization via integron-mobilization units (IMU), has been previously reported (Poirel et al., 2009).

This study aimed to evaluate the occurrence and diversity of carbapenem-resistant Gram-negative bacteria over the different treatment stages in a UWWTP

that includes UV-C disinfection. CRE, known as major carriers of mobile carbapenem-resistance genes, had a special focus in this study. These strains were characterized by whole genome sequencing and we describe the genetic environment of *bla*$_{GES-5}$ located on class 1 and class 3 integrons. This investigation contributes to assess the impact of UWWTPs in the surrounding environment and the role of these commodities as barriers or potential sources of contaminant antibiotic resistance.

3. METHODS

3.1. Sampling

Grab wastewater samples were collected in a full-scale UWWTP, located in Northern Portugal, which receives both domestic and hospital effluents (Silva et al., 2018). The wastewater treatment process includes primary decantation, activated sludge digestion and UV-C disinfection through an open channel UV system, using a 150 W lamp (Trojan). The UV dose was 29.74 mJ/cm^2, with a contact time of 11.44s. Grab samples were collected in three sampling moments between June and September, after the first settling tank (herein referred to as raw wastewater; samples RW1, RW2 and RW3 corresponding to the three sampling campaigns), after secondary treatment (SW1, SW2 and SW3 samples) and from the final effluent after UV-C disinfection (TW1, TW2 and TW3 samples). Samples were collected in sterile flasks, transported to the lab in refrigerated containers and processed within 12 h. Bacterial regrowth samples, designated TWr1, TWr2 and TWr3, corresponded to TW samples that were kept for 3 days in the dark at 20 °C.

3.2. Enumeration and isolation of culturable bacteria

Wastewater samples were homogenized and filtered through 0.45 µm-pore-size cellulose membranes (Sartorius Stedim Biotech, Germany, either directly or after being serially diluted (10-fold). Membranes were inoculated on membrane-Fecal Coliform medium (mFC, Difco, USA) without antibiotic or supplemented

with 4 mg/mL of meropenem (Sigma-Aldrich, USA). Cultures were incubated at 37°C and the number of colony-forming units (CFUs) was enumerated after 24 h. Samples were processed in triplicate. The medium and incubation temperature were selected to enumerate Gram-negative bacteria, particularly coliforms, and to reduce plasmid loss and increase the functional stability of beta-lactam resistance mechanisms, as previously described (Marano et al., 2020). Plates with a countable number of colonies (20 to 200 colonies per plate) were selected corresponding, for mFC agar, to dilutions of 10^{-4} to 10^{-5} for RW samples (depending on the sampling campaign), of 10^{-3} for ST samples and of 10^{-1} to 10^{-2} for TT samples. For mFC agar supplemented with meropenem, counted plates corresponded to dilutions of 10^{-2} (RW samples), 10^{-1} to 10^{-2} (ST samples), and the filtration of 1 to 10 mL without dilution (for TT samples).

Colonies formed on meropenem-supplemented culture medium were selected for purification by sub-culturing on mFC agar. Cultures were preserved in LB (Luria-Bertani) medium (Difco, USA) with 20% (w/v) glycerol at -80 °C.

3.3. Genotyping by rep-PCR and identification

To select non-clonal isolates for further analyses a rep-PCR typing was performed. For this, one bacterial colony was resuspended in 5 µL of sterile distilled water and 1 µL of this cell suspension was used as template in each PCR reaction. PCR reactions composition and programmes for BOX-PCR and ERIC-PCR were as previously described (Araújo et al., 2014). PCR amplification was performed using a MyCycler Thermal Cycler (Bio-Rad, USA).

Isolates displaying distinct rep-PCR profiles were identified by 16S rRNA gene sequence analysis. For this, primers 27F/1492R (5'-AGAGTTTGATCCTGGCTCAG-3'/5'-GGYTACCTTGTTAACGACTT-3') were used for amplification (Lane, 1991) and sequencing as described previously (Araújo et al., 2014). Sequence similarity searches were performed with the BLAST software at the NCBI website against the GenBank database and against the EzBioCloud database using EzTaxon (http://www.ezbiocloud.net/eztaxon).

3.4. Pulsed-field gel electrophoresis (PFGE)

PFGE was applied to assess the genotypic diversity of CRE isolates. Preparation of plugs was performed according to the PulseNet protocol available at CDC website (www.cdc.gov). For each sample, DNA in agarose plugs was digested with 50 U of *Xba*I (Thermo Scientific) at 37°C for 2 h. An electrophoretic run was performed with a CHEF-DR II apparatus (Bio-Rad) using the following conditions adapted from CDC (CDC, 2013): 6 V/cm; initial time 6.8 s; final time 35.4 s; for 17 h, at 14°C.

3.5. Antibiotic susceptibility testing

A total of 52 isolates were tested for antimicrobial susceptibility by the disk diffusion method on Mueller-Hinton Agar (Oxoid, Basingstoke, UK) according to the EUCAST guidelines (European Committee on Antimicrobial Susceptibility Testing, 2020). Discs were purchased from Oxoid (UK) and the antibiotics tested for each bacterial group were chosen according to EUCAST. All isolates were tested using the following antibiotics: ciprofloxacin (5 µg), chloramphenicol (30 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg). CRE and Gram-negative isolates were tested against amoxicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), cefotaxime (5 µg), ertapenem (10 µg), gentamicin (10 µg) and tetracycline (30 µg). In turn, CRE and *Stenotrophomonas* isolates were tested using ceftazidime (30 µg) and cefepime (30 µg). Besides the above mentioned, CRE isolates were also tested against meropenem (10 µg), imipenem (10 µg), aztreonam (30 µg), streptomycin (10 µg) and rifampicin (5 µg); and isolates affiliated with the genus *Stenotrophomonas* were tested for ticarcillin/clavulanic acid (75/10 µg) and tigecycline (15 µg). *Escherichia coli* ATCC 25922 was used as quality control. The results were interpreted according to EUCAST (2020) criteria or CLSI (Clinical and Laboratory Standards Institute, 2015) for streptomycin susceptibility test results interpretation. Isolates with growth rank between sensitive and resistant values were considered resistant for data analysis. Minimal inhibitory concentrations (MICs) were determined in Mueller-Hinton agar, for cefotaxime, ceftazidime, imipenem, ertapenem and meropenem according to EUCAST guidelines.

3.6. Detection of antibiotic resistance genes and integrons

PCR screening was performed using primers and conditions previously reported (Araújo et al., 2017; Tacão et al., 2015) to identify genes encoding beta-lactamases (*bla*$_{L1,}$ *bla*$_{VIM}$, *bla*$_{IMP,}$ *bla*$_{GES}$, *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$-like, *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$), sulphonamides resistance (*sul1* and *sul2*) and integrases (*intI*1, *intI*2 and *intI*3). In each reaction, sterile water was used as negative control and bacterial starins from the laboratory collection carrying the inspected genes were used as positive control. Negative and positive controls were included in each PCR experiment (Tacão et al., 2015; Teixeira et al., 2020). Results were confirmed by amplicon sequencing analysis.

3.7. Conjugation experiments

The transfer of plasmid-encoded carbapenem resistance was tested by the broth culture conjugation method using the azide-resistant *E. coli* J53 and the rifampicin-resistant *E. coli* CV601 as recipient strains, as previously described (Tacão et al., 2018). Transconjugants were selected on Plate Count Agar (PCA) containing meropenem (2 μg/ml) and sodium azide (100 μg/ml) or rifampicin (100 μg/mL), and the profile of the recipient was confirmed by BOX-PCR when putative transconjugants were obtained.

3.8. Statistical analysis

Differences among CFUs counts of total CRB determined for each treatment step (raw wastewater, after secondary treatment and after UV-C disinfection) were tested using ANOVA (aov function, stats package) and TukeyHSD, analyzed with dplyr (Wickham et al., 2018) and plotted using ggplot2 (Wickham, 2016). All tests were performed using stats package in R software (R Development Core Team, 2008).

3.9. Whole genome sequencing analysis

For whole genome sequencing, DNA was purified using the Wizard Genomic DNA Purification kit (Promega). Paired-end libraries were generated using

Illumina technology (GATC; Eurofins, Germany). Quality of raw reads was evaluated using the software FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and reads with a Phred quality score less than 20 were removed by applying Trimmomatic (version 0.36, parameters: illuminaclip on, slidingwindow 4:15, leading , trailing 3, crop off, minlen 36) (Bolger et al., 2014). Draft genomes were assembled with SPAdes version 3.11.0 (Bankevich et al., 2012) and annotated using RAST (Aziz et al., 2008). Contamination in the genome assembly was verified using ContEst16S (Lee et al., 2017) and CheckM (Parks et al., 2015). Species identification was confirmed by calculating for each genome and its closest relative, the digital DNA-DNA hybridization (dDDH) values using the Genome-to-Genome Distance Calculator 2.1 (Meier-Kolthoff et al., 2013) and the average nucleotide identity (ANI) numbers were calculated applying ANIb (BLAST) and ANIm (MUMmer) algorithms, with the tools available at the JSpecies WS website (jspecies.ribohost.com/jspeciesws/) (Richter et al., 2016). The G+C% divergence between each genome with the closest relative was also considered (Meier-Kolthoff et al., 2014). Since one of the isolates did not affiliate with any known species using the above mentioned parameters, the phylogenetic affiliation at species level was further investigated for all isolates through the construction of a phylogenetic tree based on *gyrB*, *rpoB* and *atpD* concatenated complete gene sequences retrieved from the genomes and from closest relative strains (Figure 2). A maximum likelihood tree was constructed using molecular MEGA 6.0 (Tamura et al., 2013) with 1000 bootstrap replications. Kimura-2-parameter was used as the model of DNA sequence evolution.

Draft genomes were analysed with tools available at the Center for Genomic Epidemiology website (www.genomicepidemiology.org), to determine the presence of resistance genes (Resfinder 3.0) and plasmids (PlasmidFinder 1.3). The resistome was further predicted by performing a search against the Comprehensive Antibiotic Resistance Database (CARD, Alcock et al., 2020; McArthur et al., 2013).

3.10. Nucleotide sequences accession numbers

Whole genome sequences were submitted to DDBJ/ENA/GenBank under the accession numbers WLVC00000000 (isolate RWM.1), WLVD00000000 (isolate RWM.4), WLVE00000000 (isolate RWM.8).

4. RESULTS

4.1. Gram-negative CRB abundance over treatment

Significant (p<0.0001) reduction of total bacterial counts in mFC agar was observed after secondary treatment and after UV-C disinfection (reduction of 1.9 and 1.8-log units, respectively; Figure 1). For bacteria counted on culture medium supplemented with meropenem, reduction was not significant in SW samples (0.2-log units; p>0.05). However, after UV-C disinfection a significant reduction of CRB was observed (2.4-log units; p<0.0001). Based on bacterial counts on medium with and without antibiotic, we estimated a prevalence of 0.1% of meropenem-resistant bacteria in RW samples. This percentage increased to 10% for SW samples but decreased again to 1% (p<0.001) after UV-C disinfection. Of note is the fact that this percentage increased to 6% after incubation of final effluent samples in the dark for 3 days (TWr).

4.2. CRE isolates in raw wastewater

Isolates from raw wastewater samples with typical *Enterobacteriaceae*-like colony morphology (shades of blue-colored colonies) were selected for further analysis. These colonies were only detected in RW samples and represented 1 to 10% of the total number of colonies in these plates. These isolates (n=34) corresponded to 17 distinct genotyping profiles obtained based on rep-PCR typing and PFGE (Figure 2) and based on the 16S rRNA gene-based identification (Table 1) were affiliated to the genera *Citrobacter* (n=11), *Enterobacter* (n=2), *Leclercia* (n=3) and *Lelliottia* (n=1). All representative isolates (n=17) were resistant to three or more classes of antibiotics (Table 1), being resistant at least

to carbapenems, cefotaxime, ciprofloxacin, rifampicin and streptomycin. In this group of isolates, common resistance phenoytpes observed in 94% (n=16) of the isolates were determined for amoxicillin, amoxicillin/clavulanic acid, ceftazidime and gentamycin. Only one isolate (*Leclercia* RWM.10) was resistant to chloramphenicol and tetracycline. Integrase genes were detected in all isolates (Table 1), namely *intI1* (in 16 isolates) and *intI2* and *intI3* (both in 15 isolates). Based on PCR analysis was detected the gene bla_{TEM} in 4 isolates, the gene bla_{GES-5} in all the isolates and the gene *sul1* in 15 isolates (Table 1). In two isolates, bla_{GES-5} was associated to class 1 integrons, and in 15 isolates to class 3 integrons.

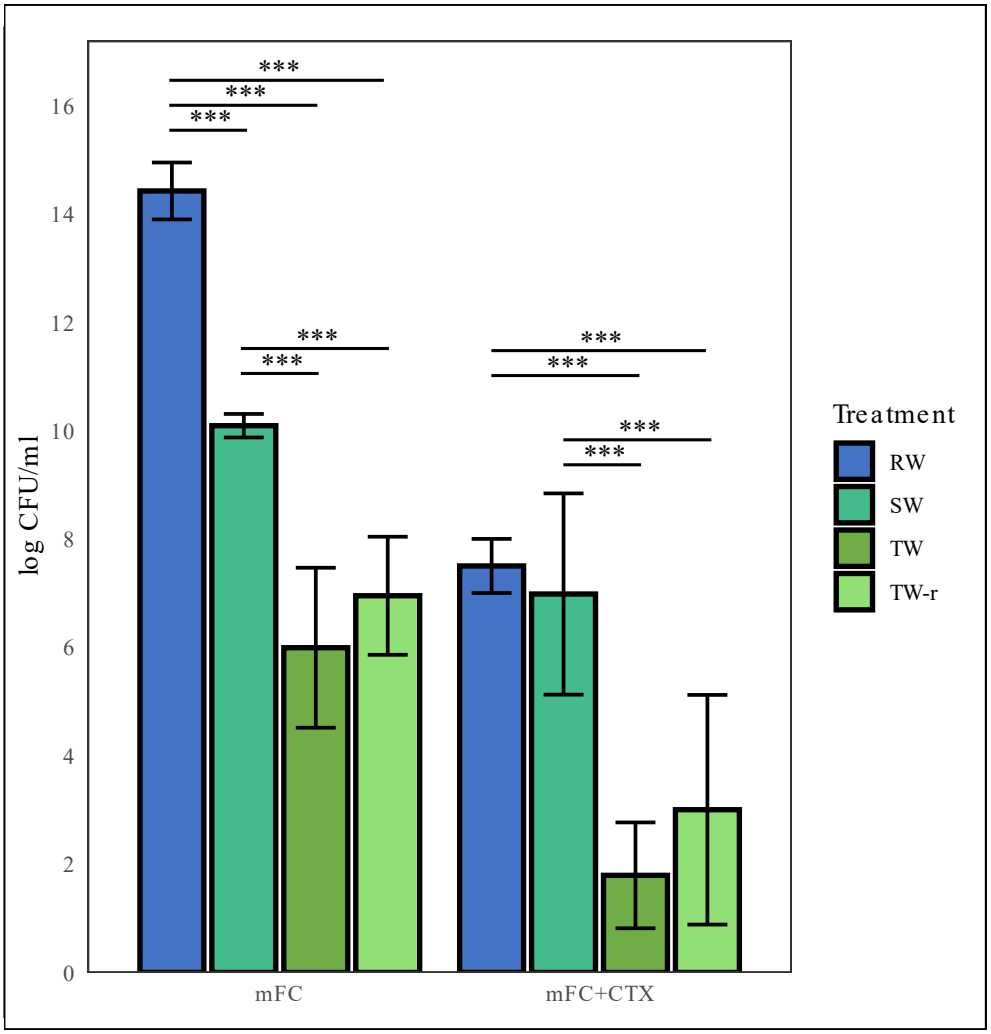


FIGURE 1. Bacterial counts of CFUs per volume of wastewater sample (mL) collected from raw water (RW; blue columns), after secondary treatment (SW; aquamarine green) and after UV-C disinfection (TW; light green). Counts for TW samples stored for 3 days at 20 °C under light protection are also presented (TW-r, very light green). Counts were obtained on plates of mFC and mFC supplemented with meropenem (mFC+MEM), incubated for 24 h at 30 °C. Statistically significant differences are shown with $p<0.0001$ (***).

TABLE 1. Phylogenetic affiliation and antibiotic resistance genotypes and phenotypes of meropenem-resistant isolates retrieved from raw wastewater (samples RW; isolates RWM), final effluent after UV-C disinfection (samples TW; isolates TWM) and after incubation in the dark for 3 days (samples TWr; isolates TWrM). Numbers of the first column correspond to the three sampling moments (1 - June, 2 - July and 3 - September).

Sampling date	Isolates	Phylogenetic affiliation[a]	ARGs[b,c]	*intI* genes	Antibiotic Resistance Phenotype[d,e]
2	RWM.1[f]	*Lelliottia* sp.	*bla*$_{TEM}$, *bla*$_{GES-5}$, *sul1*	*intI1*	AML, AMC, CTX (16), CAZ (32), FEP, MEM (32), IMI (32), ERT (12), AZT, GEN, S, CIP, SXT, RIF
2	RWM.2	*Leclercia* sp.	*bla*$_{TEM}$, *bla*$_{GES-5}$, *sul1*	*intI1*, *intI2*, *intI3*	AML, AMC, CTX, CAZ, FEP, MEM, IMI, ERT, AZT, GEN, S, CIP, SXT, RIF
2	RWM.3	*Citrobacter* sp.	*bla*$_{GES-5}$, *sul1*	*intI1*, *intI2*, *intI3*	AML, AMC, CTX, CAZ, MEM, IMI, ERT, GEN, S, CIP, SXT, RIF
2	RWM.4[f]	*Citrobacter* sp.	*bla*$_{GES-5}$, *sul1*	*intI1*, *intI2*, *intI3*	AML, AMC, CTX (16), CAZ (16), MEM (32), IMI (32), ERT (32), S, CIP, SXT, RIF
2	RWM.5	*Citrobacter* sp.	*bla*$_{GES-5}$	*intI1*, *intI2*, *intI3*	AML, AMC, CTX, CAZ, MEM, IMI, ERT, GEN, S, CIP, RIF
2	RWM.6	*Citrobacter* sp.	*bla*$_{GES-5}$, *sul1*	*intI1*, *intI2*, *intI3*	AML, AMC, CTX, CAZ, FEP, MEM, IMI, ERT, GEN, S, CIP, SXT, RIF
2	RWM.7	*Citrobacter* sp.	*bla*$_{GES-5}$, *sul1*	*intI1*, *intI2*, *intI3*	AML, AMC, CTX, CAZ, MEM, IMI, ERT, AZT, GEN, S, CIP, SXT, RIF
2	RWM.8[f]	*Citrobacter* sp.	*bla*$_{GES-5}$	*intI2*, *intI3*	AML, AMC, CTX (8), CAZ (16), FEP, MEM (32), IMI (32), ERT (32), GEN, S, CIP, RIF
2	RWM.9	*Citrobacter* sp.	*bla*$_{GES-5}$, *sul1*	*intI1*, *intI2*, *intI3*	AML, AMC, CTX, CAZ, FEP, MEM, IMI, ERT, AZT, GEN, S, CIP, SXT, RIF
2	RWM.10	*Leclercia* sp.	*bla*$_{TEM}$, *bla*$_{GES-5}$, *sul1*	*intI1*, *intI2*, *intI3*	AML, AMC, CTX, CAZ, FEP, MEM, IMI, ERT, AZT, GEN, S, CIP, C, TE, SXT, RIF
2	RWM.11	*Citrobacter* sp.	*bla*$_{GES-5}$, *sul1*	*intI1*, *intI2*, *intI3*	AML, AMC, CTX, CAZ, FEP, MEM, IMI, ERT, AZT, GEN, S, CIP, SXT, RIF
2	RWM.12	*Citrobacter* sp.	*bla*$_{GES-5}$, *sul1*	*intI1*, *intI2*, *intI3*	CTX, CAZ, FEP, MEM, IMI, ERT, AZT, GEN, S, CIP, SXT, RIF
2	RWM.13	*Enterobacter* sp.	*bla*$_{GES-5}$, *sul1*	*intI1*, *intI2*, *intI3*	AML, AMC, CTX, CAZ, FEP, MEM, IMI, ERT, AZT, GEN, S, CIP, SXT, RIF
2	RWM.14	*Citrobacter* sp.	*bla*$_{GES-5}$, *sul1*	*intI1*, *intI2*, *intI3*	AML, AMC, CTX, MEM, IMI, ERT, GEN, S, CIP, SXT, RIF
2	RWM.15	*Citrobacter* sp.	*bla*$_{GES-5}$, *sul1*	*intI1*, *intI2*, *intI3*	AML, AMC, CTX, CAZ, MEM, IMI, ERT, GEN, S, CIP, SXT, RIF
2	RWM.16	*Enterobacter* sp.	*bla*$_{GES-5}$, *sul1*	*intI1*, *intI2*, *intI3*	AML, AMC, CTX, CAZ, FEP, MEM, IMI, ERT, AZT, GEN, S, CIP, SXT, RIF
2	RWM.17	*Leclercia* sp.	*bla*$_{TEM}$, *bla*$_{GES-5}$, *sul1*	*intI1*	AML, AMC, CTX, CAZ, FEP, MEM, IMI, ERT, AZT, GEN, S, CIP, SXT, RIF
1	TWM.1	*Stenotrophomonas maltophilia*	*bla*$_{L1}$	-	CAZ, FEP, CIP
1	TWM.2	*S. maltophilia*	*bla*$_{L1}$	-	TIM, CIP
1	TWM.3	*S. maltophilia*	*bla*$_{L1}$	-	CAZ, CIP
1	TWM.4	*S. maltophilia*	*bla*$_{L1}$	-	CAZ, FEP, CIP, C
2	TWM.5	*S. maltophilia*	*bla*$_{L1}$	-	CIP
2	TWM.6	*S. maltophilia*	*bla*$_{L1}$	-	CAZ, FEP, TIM, CIP, C
2	TWM.7	*S. maltophilia*	*bla*$_{L1}$	-	n.d.
2	TWM.8	*S. maltophilia*	*bla*$_{L1}$	-	CIP
3	TWM.9	*S. maltophilia*	*bla*$_{L1}$	-	CAZ, FEP, CIP
3	TWM.10	*S. maltophilia*	*bla*$_{L1}$	-	n.d.

3	TWM.11	*S. maltophilia*	bla_{L1}	-	CIP
3	TWM.12	*S. maltophilia*	bla_{L1}	-	CIP
1	TWrM.13	*S. maltophilia*	bla_{L1}	-	FEP, CIP
1	TWrM.14	*S. maltophilia*	bla_{L1}	-	CIP
1	TWrM.15	*S. maltophilia*	bla_{L1}	-	CIP, C
2	TWrM.16	*S. maltophilia*	bla_{L1}	-	CAZ, CIP, C
2	TWrM.17	*S. maltophilia*	bla_{L1}	-	CIP, C
3	TWrM.18	*S. maltophilia*	bla_{L1}	-	CAZ, FEP, TIM, C
3	TWrM.19	*S. maltophilia*	bla_{L1}	-	CAZ, FEP, TIM, CIP, C
3	TWrM.20	*S. maltophilia*	bla_{L1}	-	CAZ, FEP, TIM, CIP, C
3	TWrM.21	*S. maltophilia*	bla_{L1}	-	CAZ, FEP, TIM, CIP, C, TGC
3	TWrM.22	*S. maltophilia*	bla_{L1}	-	CAZ, FEP, TIM, CIP, C
3	TWrM.23	*S. maltophilia*	bla_{L1}	-	CAZ, FEP, CIP, C
3	TWrM.24	*S. maltophilia*	bla_{L1}	-	CAZ, FEP, CIP, C
3	TWrM.25	*S. maltophilia*	bla_{L1}	-	CIP
1	TWrM.1	*Pandorea pnomenusa*	-	-	AML, CTX, ERT
1	TWrM.2	*Chryseobacterium rhizoplanae*	-	-	AML, AMC, CTX, ERT, GEN
1	TWrM.3	*Pandorea norimbergensis*	-	-	AML, AMC, CTX, ERT, GEN, CIP
1	TWrM.4	*Cupriavidus pauculus*	-	-	ERT, GEN, CIP
1	TWrM.5	*C. pauculus*	-	-	ERT, GEN, CIP
1	TWrM.6	*P. pnomenusa*	-	-	AML, AMC, CTX, ERT, GEN
1	TWrM.7	*Ralstonia pickettii*	-	-	AML, AMC, CTX, ERT, GEN, C
3	TWrM.8	*C. pauculus*	-	-	ERT, GEN, C
3	TWrM.9	*C. pauculus*	-	-	ERT, GEN, C
3	TWrM.10	*C. pauculus*	-	-	AML, AMC, ERT, GEN

[a]According to 16S rRNA gene sequence comparison against EzBioCloud (http://www.ezbiocloud.net/eztaxon); for *Enterobacteriaceae* only genus affiliation is presented due to the low resolution of 16S rRNA gene-based affiliation.
[b]ARG (Antibiotic Resistance Genes) detected by PCR.
[c]-, genes not detected.
[d]Antibiotics: AML - amoxicillin, AMC - amoxicillin/clavulanic acid, TIM - ticarcillin/clavulanic acid, CTX - cefotaxime, CAZ - ceftazidime, FEP - cefepime, MEM - meropenem, IMI - imipenem, ERT - ertapenem, CIP - ciprofloxacin, C - chloramphenicol, TE - tetracycline, TGC - tigecycline, GEN - gentamicin, SXT - trimethoprim/sulfamethoxazole, TET - tetracycline.
[e]n.d., not determined due to the fact that the isolates did not grow in Mueller Hinton Agar.
[f]Isolates for which genomes were sequenced.

Three isolates representing distinct PFGE profiles (Figure 2) were selected for whole genome sequencing: *Lelliottia* RWM.1 and *Citrobacter* RWM.4 and RWM.8. For these isolates, MICs between 12 and 32 µg/mL for carbapenems and of 8 to 32 µg/mL for 3rd generation cephalosporins were determined (Table 1).

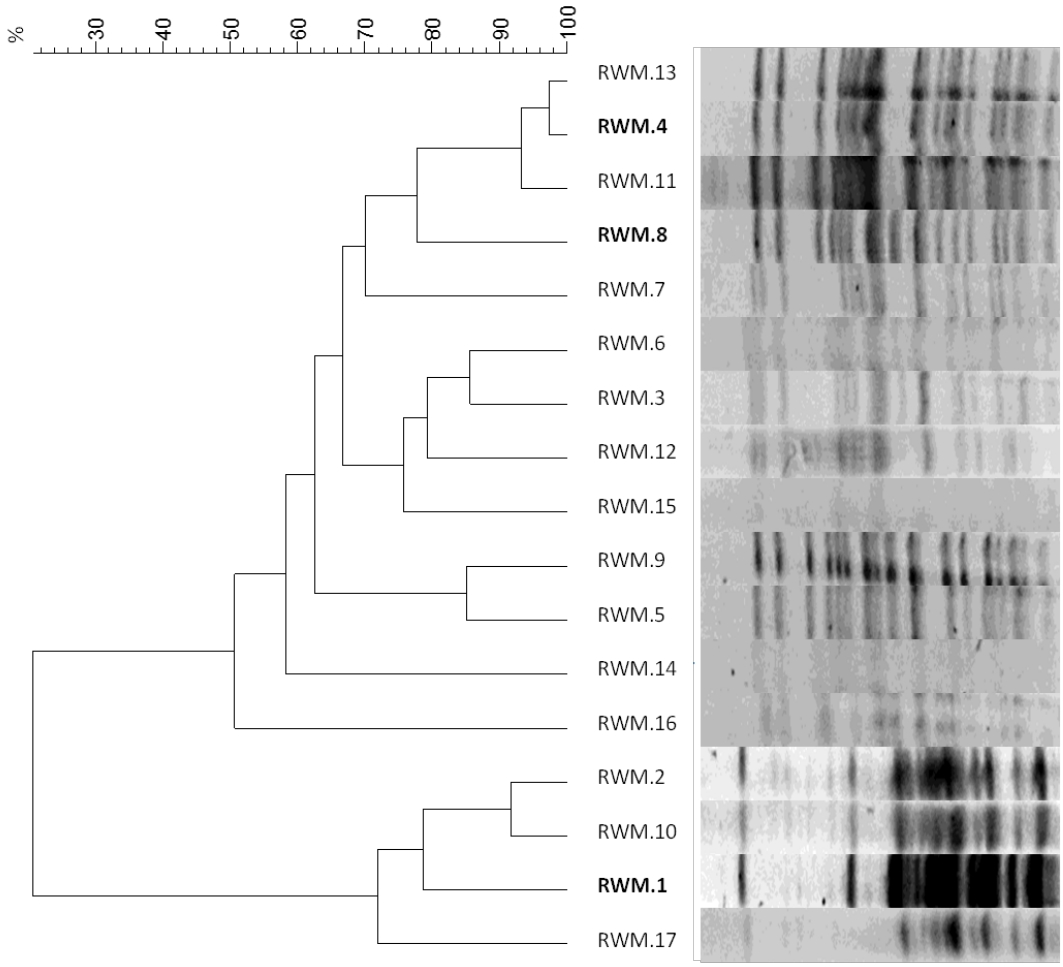


FIGURE 2. PFGE profiles of CRE isolates digested with *Xba*I and analysed using GelComparII. Isolates selected for whole genome sequencing are highlighted in bold.

Genomes general features are presented in Table S1. CheckM (Parks et al., 2015) predicted low levels of contamination in the genomes (Table S1) and no foreign 16S rRNA gene fragments were detected. Genome sequence-based methods [i.e. average nucleotide identity (ANIb) and digital DNA-DNA hybridization (DDH) calculation] were congruent in the identification of *Citrobacter* isolates as *Citrobacter braakii* (Table S2). This affiliation is also evident

from the maximum likelihood phylogenetic reconstruction using genes *gyrB*, *rpoB* and *atpD* (Figure 3). Regarding isolate *Lelliottia* sp. RWM.1, ANI values below 95% and DDH below 70% between this isolate and type strains of the closest related species, suggest it may be a novel species (Chun et al., 2018). The phylogenetic analysis (Figure 3) confirms *Lelliottia jeotgali* and *Lelliottia amnigena* as *Lelliottia* sp. RWM.1 nearest neighbours.

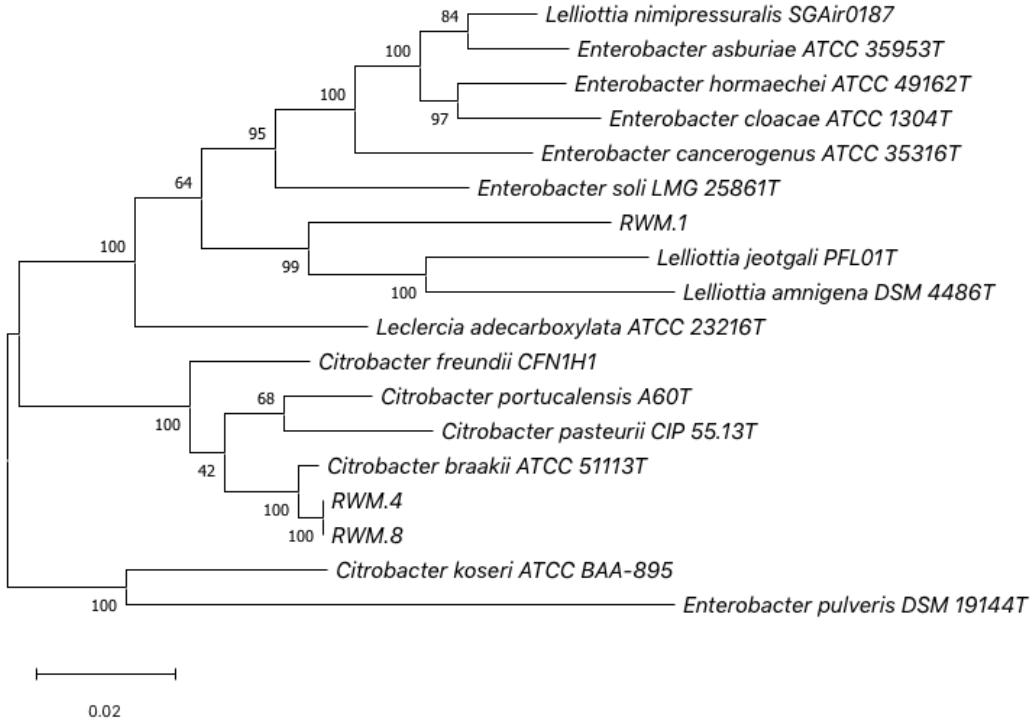


FIGURE 3. Maximum likelihood tree (Tamura-Nei model) based on concatenated complete *gyrB*, *rpoB* and *atpD* gene sequences retrieved from isolates obtained in this study with sequenced genomes (RWM.1, RWM.4, RWM.8) and from strains representing closely related species included in genera *Lelliottia* (*Lelliottia amnigena* DSM 4486^T - GenBank accession no. PDDA01000000, *Lelliottia jeotgali* PFL01^T - CP018628, *Lelliottia nimipressuralis* SGAir0187 - CP025034), *Enterobacter* (*Enterobacter asburiae* ATCC 35953^T - CP011863, *Enterobacter cancerogenus* ATCC 35316 - ABWM00000000, *Enterobacter cloacae* ATCC 13047^T - CP001918, *Enterobacter hormaechei* ATCC 49162^T - MKEQ00000000, *Enterobacter pulveris* DSM 19144^T - JHYZ00000000, *Enterobacter soli* LMG 25861^T - FYBB00000000), *Leclercia* (*Leclercia adecarboxylata* ATCC 23216^T - BCNP01000000) and *Citrobacter* (*Citrobacter braakii* ATCC 51113^T - NAEW00000000, *Citrobacter freundii* CFNIH1 - CP007557, *Citrobacter koseri* ATCC BAA-895 - CP000822, *Citrobacter pasteurii* CIP 55.13^T - CDHL00000000, *Citrobacter portucalensis* A60^T - MVFY00000000). Bootstrap values are indicated in % at nodes. Branch lengths represent the number of substitutions per site.

Consistent with the described multidrug resistance phenotypes, *in silico* resistome analysis (Table 2) detected the presence of genes conferring resistance to 9 distinct antibiotic classes, some of which were also detected by PCR. In general *C. braaki* isolates harbour similar resistance determinants, comprising genes encoding beta-lactamases (i.e. bla_{GES-5} and $bla_{CMY-101}$), resistance to aminoglycosides (e.g. *aadA1*), fluoroquinolones (*qnrB10*), trimethoprim (*dfrA1*) and streptothricin (*sat2*). Additionally, in *C. braakii* RWM.4 were also present the genes *aacA4* (resistance to aminoglycosides), *sul1* (resistance to sulphonamides), *arr3* (resistance to rifampicin and *mph*(*A*) (erythromycin resistance). *Lelliottia* sp. RWM.1 presented a distinct resistome, e.g. with additional beta-lactamase genes and chloramphenicol resistance determinant, whereas rifampicin or streptothricin resistance determinants were not detected.

TABLE 2. Antibiotic resistance genes, plasmids and integrase genes identified in all sequenced genomes.

		Isolates		
		RWM.1	**RWM.4**	**RWM.8**
Antibiotic resistance genes	Beta-lactams	bla_{GES-5} bla_{BEL-1} bla_{OXA-1} bla_{TEM-1B} bla_{DHA-1}	bla_{GES-5} $bla_{CMY-101}$	bla_{GES-5} $bla_{CMY-101}$
	Aminoglycosides	bla_{OXA}/*aacA4'* *strA*	bla_{OXA}/*aacA4'* *aacA4*-like *aadA1*	bla_{OXA}/*aacA4'* *aadA1*
	Fluoroquinolones	*qnrB4*	*qnrB10*	*qnrB10*
	Macrolides	*ere(A)* *mph(A)*	*mph(A)*	-
	Phenicol	*catB3*	-	-
	Sulphonamides	*dfrA19*	*dfrA1*	*dfrA1*
	Trimethoprim	*sul1*	*sul1*	-
	Streptothricin	-	*sat2*	*sat2*
	Rifampicin	-	*arr-3*	-
Plasmids		IncFII (Yp), IncHI2, IncHI2A, IncX6, IncP6, IncFIB (K), col. 4401	IncFII (Yp) IncQ2 RepA	IncQ2
Integrase genes		*intI1*	*intI1*, *intI2*, *intI3*	*intI2*, *intI3*

Plasmids were predicted in all genomes ranging from one replicon in RWM.8 (IncQ2, which was common to all *C. braaki* genomes) to seven distinct replicons predicted in *Lelliottia* sp. RWM.1. In accordance with PCR results, integrons were also detected in all genomes (Table 2).

The genomic context of the gene bla_{GES-5} was analysed (Figure 4). In *Lelliottia* sp. RWM.1 the gene was detected on a 4,048 bp contig, as part of a class 1 integron. A BLAST search revealed that 65% of this contig shared 100% identity with plasmid pKPN1144 (GenBank accession number KF745070), a ColE1 plasmid from *Klebsiella pneumoniae*. Downstream *intI1*, three open reading frames were found displaying 100% similarity with previously described gene cassettes: the bla_{OXA}/*aacA4* fusion, bla_{GES-5} and bla_{BEL-1}. Conserved *attI* and *attC* sequences were detected following the integrase gene and gene cassettes, respectively. A putative hybrid PcH1 promoter (TGGACA – TAAACT) was located within *intI1* followed by a P2 promoter (TTGTTA – TACAGT). Despite the use of several primer combinations it was not possible to further assemble this novel integron. Conjugation assays were unsuccessful for this strain.

In *C. braaki* RWM.4 bla_{GES-5} was detected in a contig twith 24,715 bp (Figure 4). About 50% of the contig shared 99.7% similarity with the plasmid pKOR-e3cb of *Klebsiella oxytoca* (GenBank accession number CP026282), corresponding to a region with several transposases genes related to the nitrogen cycle. In our isolate, this region was followed by a class 1 integron with an *aacA4* gene cassette and lacking the 3'CS conserved region, and by a class 3 integron 100% identical to the one described for *C. braakii* RWM.8, including the bla_{GES-5} cassette and 89 nucleotides of bla_{OXA-10}/*aacA4*. Due to high similarity to known plasmids the mobile potential of the plasmids was evaluated through mating assays, but no transconjugants were obtained.

In *C. braaki* RWM.8, bla_{GES-5} was detected in a contig that corresponds to the whole sequence of an IncQ plasmid (pRWM8) with 10,482bp (Figure 4). Hence, the plasmid replication origin and iterons, replication genes and a putative mobilization module including a *nic* site, were identified in pRWM8 sequence. Replication and mobilization genes were 100% identical to the ones found in plasmids pQ7 from *E. coli* (GenBank accession number FJ696404), pCR16 from *Citrobacter freundii* (RBWI00000000; Teixeira et al., 2020), pJF-789 from

Klebsiella oxytoca (KX912254) and pPCM13 from *Serratia marcescens* (MH569711). pRWM8 carried a class 3 integron with two gene cassettes (*bla*$_{GES-5}$-*bla*$_{OXA-10}$/*aacA4*) for which the *attC* motifs were identified. The integrase gene (*intI3*) was disrupted by a putative transposase inserted in the Pc promoter region (TAGACA – TAGGCT), which was 92% identical to a transposable element of the IS*256* family previously reported in *Azoarcus* sp. (GenBank accession number CR555308). A conserved integrase promoter was identified downstream the transposase gene as well as the *attI* region. pRWM8 lacked conjugation genes, which is in accordance with the negative results obtained in mating assays.

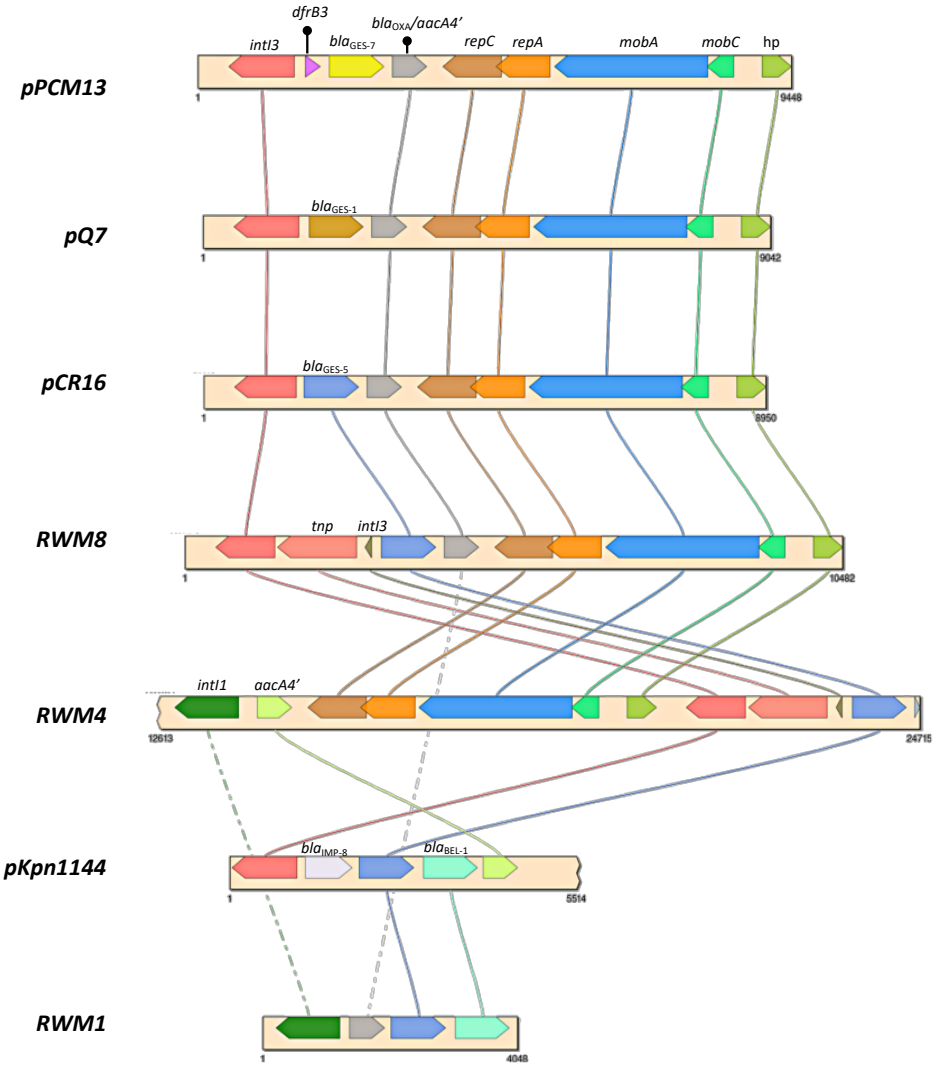


FIGURE 4. Synteny analysis of *bla*$_{GES-5}$ gene context in plasmid pPCM13 of *S. marcescens* (GenBank accession no. MH569711), pQ7 of *E. coli* (GenBank accession no. FJ696404), pCR16 (GenBank accession no. RBWI00000000) pKPN1144 of *K. pneumoniae* (GenBank

accession no. KF745070) and in isolates RWM.8, RWM.4 and RWM.1; Lines indicate conserved genes.

4.3. Carbapenem-resistant Gram-negative bacteria in TW and TW-r samples

Isolates with typical *Enterobacteriaceae*-like colony morphology were not detected after secondary treatment nor after UV-C disinfection. In order to assess which other Gram-negative bacteria were present after UV-C disinfection, a subset of 60 isolates were randomly selected for further analysis, being picked 10 from each sample type (TW or TW-r) of each campaign. Isolates were subjected to BOX-PCR and ERIC-PCR fingerprint analysis. A total of 35 unique profiles were obtained (data not shown). Representatives of each typing profile were identified based on 16S rRNA gene sequence analysis (Table 1). Twenty-five isolates affiliated with *Stenotrophomonas maltophilia* (Class *Gammaproteobacteria*; Family *Xanthomonadaceae*). For those, identification was confirmed through the amplification of the species-specific gene bla_{L1}. The remaining 10 representative isolates affiliated with *Cupriavidus pauculus* (*n*=5; *Betaproteobacteria*; *Burkholderiaceae*), *Pandoraea pnomenusa* (*n*=2; *Betaproteobacteria*; *Burkholderiaceae*), *Pandoraea norimbergensis* (*n*=1; *Betaproteobacteria*; *Burkholderiaceae*), *Ralstonia pickettii* (*n*=1; *Betaproteobacteria*; *Burkholderiaceae*), and *Chryseobacterium rhizoplanae* (*n*=1; *Flavobacteria*; *Flavobacteriaceae*).

The *S. maltophilia* isolates were resistant to most tested antibiotics, with resistance levels of 88% to ciprofloxacin, 52% to ceftazidime, 48% to cefepime and chloramphenicol, and 28% to the combination ticarcillin/clavulanic acid. One isolate (TWrM21; Table 1) was resistant to tigecycline. All isolates were susceptible to the combination trimethoprim/sulfamethoxazole. Multidrug resistance was observed in 10 isolates (40%). Isolates that affiliated with other genera were resistant to beta-lactams including, as expected, ertapenem (100% of the isolates) but also amoxicillin (60%), amoxicillin/clavulanic acid (50%) and cefotaxime (50%). These isolates were also frequently resistant to gentamycin (70%), chloramphenicol (50%) and resistance to ciprofloxacin was also detected (10%). Fifty percent of these isolates were multiresistant. Apart from the bla_{L1}

gene detected in all *S. maltophilia* isolates, no other ARG was detected in isolates from TW and TW-r samples.

Considering the high prevalence of *S. maltophilia* in the subset of isolates analysed (n=60), the whole collection from TW and TWr samples (n=895 isolates; isolated from TW and TW-r samples and conserved at -80ºC) was further screened for the presence of the species-specific gene bla_{L1}. A total of 683 isolates (76.3%) were positive for this gene.

5. DISCUSSION

Increasing levels of resistance to carbapenems are a global concern given the importance of these compounds as antibiotics of last resort. In this study we evaluated the prevalence of CRB throughout a UWWTP, which includes a final disinfection step with UV-C radiation. We used a selective medium (mFC, Difco) that has been proposed for the enumeration of fecal coliforms at 44°C. However, previous studies demonstrated that incubation of mFC plates at 37°C, as followed in this study, allows the enumeration of other Gram-negative bacteria, particularly coliforms of the *Enterobacteriaceae* family (Marano et al., 2020; Silva et al., 2018).

Low prevalence values were recorded in raw water when compared to levels of resistance to other clinically relevant antibiotics (e.g. Silva et al., 2018). This resulte may be related with a restrictive use of carbapenems in Portugal (PPCIRA, 2017). Although the secondary treatment significantly reduced the abundance of cultured bacteria (1.9-log units; $p<0.0001$), the prevalence of meropenem-resistant bacteria did not suffer a significant variation (0.2-log units; $p<0.05$), suggesting a positive selection already reported in other studies (Hrenovic et al., 2017b). Since many UWWTPs rely solely on secondary treatment (European Environment Agency, 2017, 2016; Ministério do Ambiente e Transição Energética, 2019), this finding is of great concern. On the other hand, according to our data, UV-C disinfection increased the quality of the final effluent, as had already been reported when evaluating the efficacy of this treatment in removing bacteria resistant to other antibiotics such as 3rd generation cephalosporins (Silva et al., 2018). These results, however, should be interpreted with caution since data was obtained exclusively through culture-dependent analysis.

Furthermore, we have to point out that the prevalence of CRB after this treatment was still slightly higher than in raw water (1% vs. 0.1%) and increased after incubation of the final effluent samples in the dark.

The quantitative data presented here may have been affected to some extent by the sampling strategy, since grab sampling captures bacterial concentrations only at the point of time in which the sample is collected. However, the results obtained in three independent sampling campaigns confirmed the general trend, thus supporting the conclusions and providing insights in the treatment performance in removing CRB. Even so, in future research composite sampling should be preferred over grab sampling, whenever possible, to further confirm the fate of these bacteria along the wastewater treatment process and to assess temporal fluctuations.

CRE pose a serious threat to public health, as carbapenems are often the last therapeutic option to treat infections caused by pathogens included in this family. In this study we found CRE in low amounts and only in raw water which suggests the efficacy of the wastewater treatment in eliminating these bacteria. However, although sporadically, CREs have been detected in final UWWTP effluents even after application of advanced treatments (Lamba and Ahammad, 2017; Yang et al., 2016). In addition, studies using metagenomics and/or qPCR approaches have shown the presence of genes that encode clinically-relevant carbapenemases, typical of *Enterobacteriaceae*, in these effluents (Lamba and Ahammad, 2017; Yang et al., 2016). These reports suggest that under certain conditions a part of the CRE present in raw wastewater may survive the treatment and be released into the environment. Thus, the occurrence of CRE in raw water is in itself a warning sign, justifying a detailed characterization of these isolates.

CRE strains here detected belong to pathogenic species of human concern such as *C. braakii* (Oyeka and Antony, 2017). All the isolates carried the carbapenemase gene bla_{GES-5}. The occurrence of GES-5-producing CREs is surprising since so far this enzyme is very rarely reported in Portuguese hospitals (Aires-de-Sousa et al., 2019; Manageiro et al., 2015a; Papagiannitsis et al., 2015). On the other hand, bla_{KPC} is the most frequent carbapenemase gene among CRE clinical isolates in Portugal (Grundmann et al., 2017), being associated with serious outbreaks (Manageiro et al., 2018; Vubil et al., 2017). Nonetheless, bla_{KPC}

was not detected in our study. In line with our findings, *bla*$_{GES-5}$ had already been detected in aquatic environments in Portugal (Manageiro et al., 2014a; Teixeira et al., 2020) and in wastewater in other countries (Girlich et al., 2012; Runcharoen et al., 2017; White et al., 2016). This apparent disparity between the resistome of clinical and wastewater environments may be due to inefficient detection of *bla*$_{GES-5}$ in clinical routine analysis, given the low levels of carbapenem resistance conferred by the expression of this gene (Vourli et al., 2004), or the inadequacy of currently used phenotypic tests (Campana et al., 2017; Pancotto et al., 2018; Sakkas et al., 2019; Tijet et al., 2013). On the other hand, we cannot exclude a non-human origin of the detected isolates since the UWWTP analysed receives wastewater from several sources (Silva et al., 2018).

Analysis of the genomic context of *bla*$_{GES-5}$ showed that isolates carried this gene as part of integrons, in several cases on mobilizable plasmids. These results are in agreement with previous reports that confirmed integrons as important platforms for the mobilization and expression of antibiotic resistance genes, gathering distinct determinants and thus contributing to multidrug resistance phenotypes (Gillings, 2014; Henriques et al., 2006; Moura et al., 2014, 2007). Of note is the co-occurrence of *bla*$_{GES-5}$ and *bla*$_{BEL-1}$ in a novel class 1 integron. *bla*$_{BEL-1}$ encodes an extended-spectrum beta-lactamase conferring resistance to 3rd generation cephalosporins, which was originally detected in *Pseudomonas aeruginosa* (Poirel et al., 2005) and only sporadically in *Enterobacteriaceae* (Papagiannitsis et al., 2015). As previously suggested, the capture of *bla*$_{GES-5}$ by integrons that are common in environmental *Enterobacteriaceae* plasmids further facilitates the spread of this gene cassette (Girlich et al., 2012). Even if *bla*$_{GES-5}$-carrying plasmids characterized in this study are not self-transmissible, the association of this gene with integrons and mobilizable plasmids is *per se* concerning since UWWTPs are acknowledge hotspots of gene exchange, where environmental bacteria and pathogens may acquire new resistance determinants (Picão et al., 2013). Although *bla*$_{GES-5}$ has been associated with low levels of carbapenem resistance, for the isolates described here high MICs were determined, possibly due to the co-expression of other resistance mechanisms such as the overexpression of efflux-pumps and downregulation or modification of porins (Beceiro et al., 2011; Fernández and Hancock, 2012; Nicolas-Chanoine et al., 2018).

Colonies with a morphology typical of non-*Enterobacteriaceae* Gram-negative bacteria were dominant (>90% in RW samples and 100% in SW, TW and TW-r samples) after all treatment stages. To better understand the phylogeny of these CRB and specially to assess the identity of the CRB released in the final effluent, we analysed a subset of isolates obtained from TW and TW-r samples. Most of these isolates belong to phylogenetic groups intrinsically resistant to carbapenems. In fact, *S. maltophilia* was the dominant species in these samples. This species produces the intrinsic L1 carbapenemase encoded in a large plasmid (Zhang et al., 2000). Although the dispersion of this resistance determinant to other bacterial species was never reported, the apparent selection of this bacterium in UWWTPs may pose a risk to human health (Kim et al., 2018). In fact, *S. maltophilia* is ubiquitous in the environment but has emerged as an important opportunistic pathogen causing hospital- and community-acquired infections in immunocompromised patients (Adegoke et al., 2017; Brooke, 2012; Wang et al., 2014), with high mortality rates (Falagas et al., 2009; Juhász et al., 2014). Besides exhibiting high levels of intrinsic antibiotic resistance, *S. maltophilia* can also acquire resistance, including fluoroquinolones, aminoglycosides, and other β-lactam antibiotics (Bostanghadiri et al., 2019). This seems to be the case of the isolates retrieved in this study, which were frequently multiresistant.

Besides *S. maltophilia*, *Chryseobacterium*, *Cupriavidus*, *Pandorea* and *Ralstonia* isolates were obtained from the final effluent. The production of class A, B and D intrinsic carbapenemases has been described for these bacteria (Bellais et al., 1999; Girlich et al., 2004; Schneider et al., 2006), and their sporadic detection in wastewater has been reported (Mir-Tutusaus et al., 2019; Reinke et al., 2020; Zhang et al., 2020). Although these CRB are mostly environmental, some were previously associated to human infections. For instance, multidrug resistant strains of *Pandoraea* have been isolated from patients suffering cystic fibrosis (Pither et al., 2020).

6. CONCLUSION

Gram-negative CRB were cultivated from wastewater, after all treatment stages in a UWWTP. Contrarily to the secondary treatment, UV-C radiation significantly reduced the abundance and prevalence of culturable CRB. We detected CRE in raw wastewater, which may be seen as an indication of the dispersion of CRE among humans or other settings subjected to high selective pressures (e.g. animal production facilities). Surprisingly the acquired carbapenemase gene here detected in CRE was *bla*$_{GES-5}$, rarely detected in Portuguese hospitals. Nevertheless, since the UWWTP receives receives wastewater from several sources, we cannot exclude a non-human origin of *bla*$_{GES-5}$-producing strains. The gene was associated with plasmids and class 1 and class 3 novel integrons, highlighting the role of these platforms in antibiotic resistance spread. The wastewater treatments applied (including UV-C disinfection) seems effective in removing CRE, since carbapenem resistance in the final effluent was only detected in other bacterial groups, most with intrinsic resistance mechanisms such as the emergent pathogen *S. maltophilia*. However, efforts to monitor UWWTPs and receiving ecosystems should be strengthened to anticipate the possible effect of such discharges in the dispersion of carbapenem resistance. This surveillance may complement the data obtained from clinical settings, allowing more comprehensive and effective actions to interrupt the spread of CRE.

7. ACKNOWLEDGEMENTS

The authors acknowledge the financial support provided by FCT (Fundação para a Ciência e a Tecnologia) through project "StARE: Stopping Antibiotic Resistance Evolution" (WaterJPI/0002/2013), CESAM funds (UIDP/50017/2020+ UIDB/50017/2020) and individual grants to Marta Tacão (CEECIND/01304/2017) and Susana Araújo (SFRH/BD/52573/2014).

8. REFERENCES

Adegoke, A.A., Stenström, T.A., Okoh, A.I., 2017. *Stenotrophomonas maltophilia* as an emerging ubiquitous pathogen: Looking beyond contemporary antibiotic therapy. Front. Microbiol. 8, 2276. doi:10.3389/fmicb.2017.02276

Aires-de-Sousa, M., Ortiz de la Rosa, J.M., Gonçalves, M.L., Pereira, A.L., Nordmann, P., Poirel, L., 2019. Epidemiology of carbapenemase-producing *Klebsiella pneumoniae* in a hospital, Portugal. Emerg. Infect. Dis. 25, 1632–1638. doi:10.3201/eid2509.190656

Alcock, B.P., Raphenya, A.R., Lau, T.T.Y., Tsang, K.K., Bouchard, M., Edalatmand, A., Huynh, W., Nguyen, A.L. V., Cheng, A.A., Liu, S., Min, S.Y., Miroshnichenko, A., Tran, H.K., Werfalli, R.E., Nasir, J.A., Oloni, M., Speicher, D.J., Florescu, A., Singh, B., Faltyn, M., Hernandez-Koutoucheva, A., Sharma, A.N., Bordeleau, E., Pawlowski, A.C., Zubyk, H.L., Dooley, D., Griffiths, E., Maguire, F., Winsor, G.L., Beiko, R.G., Brinkman, F.S.L., Hsiao, W.W.L., Domselaar, G. V, McArthur, A.G., 2020. CARD 2020: Antibiotic resistome surveillance with the comprehensive antibiotic resistance database. Nucleic Acids Res. 48, D517–D525. doi:10.1093/nar/gkz935

Araújo, S., A.T. Silva, I., Tacão, M., Patinha, C., Alves, A., Henriques, I., 2017. Characterization of antibiotic resistant and pathogenic *Escherichia coli* in irrigation water and vegetables in household farms. Int. J. Food Microbiol. 257, 192–200. doi:10.1016/j.ijfoodmicro.2017.06.020

Araújo, S., Henriques, I.S., Leandro, S.M., Alves, A., Pereira, A., Correia, A., 2014. Gulls identified as major source of fecal pollution in coastal waters: A microbial source tracking study. Sci. Total Environ. 470–471, 84–91. doi:10.1016/j.scitotenv.2013.09.075

Aziz, R.K., Bartels, D., Best, A., DeJongh, M., Disz, T., Edwards, R. a, Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R. a, McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., Zagnitko, O., 2008. The RAST Server: Rapid annotations using subsystems technology. BMC Genomics 9, 75. doi:10.1186/1471-2164-9-75

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2012. SPAdes: A new genome assembly algorithm and its applications to single-cell

sequencing. J. Comput. Biol. 19, 455–477. doi:10.1089/cmb.2012.0021

Barrios, H., Garza-Ramos, U., Ochoa-Sanchez, L.E., Reyna-Flores, F., Rojas-Moreno, T., Morfin-Otero, R., Rodriguez-Noriega, E., Garza-Gonzalez, E., Gonzalez, G., Volkow, P., Cornejo-Juarez, P., Silva-Sanchez, J., 2012. A plasmid-encoded class 1 integron contains GES-type extended-spectrum β-lactamases in *Enterobacteriaceae* clinical isolates in Mexico. Antimicrob. Agents Chemother. 56, 4032–4034. doi:10.1128/AAC.05980-11

Beceiro, A., Maharjan, S., Gaulton, T., Doumith, M., Soares, N.C., Dhanji, H., Warner, M., Doyle, M., Hickey, M., Downie, G., Bou, G., Livermore, D.M., Woodford, N., 2011. False extended-spectrum β-lactamase phenotype in clinical isolates of *Escherichia coli* associated with increased expression of OXA-1 or TEM-1 penicillinases and loss of porins. J. Antimicrob. Chemother. 66, 2006–2010. doi:10.1093/jac/dkr265

Bellais, S., Léotard, S., Poirel, L., Naas, T., Nordmann, P., 1999. Molecular characterization of a carbapenem-hydrolysing beta-lactamase from *Chryseobacterium* (*Flavobacterium*) *indologenes*. FEMS Microbiol. Lett. 171, 127–132. doi:10.1111/j.1574-6968.1999.tb13422.x

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. doi:10.1093/bioinformatics/btu170

Bostanghadiri, N., Ghalavand, Z., Fallah, F., Yadegar, A., Ardebili, A., Tarashi, S., Pournajaf, A., Mardaneh, J., Shams, S., Hashemi, A., 2019. Characterization of phenotypic and genotypic diversity of *Stenotrophomonas maltophilia* strains isolated from selected hospitals in Iran. Front. Microbiol. 10, 1191. doi:10.3389/fmicb.2019.01191

Brooke, J.S., 2012. *Stenotrophomonas maltophilia*: An emerging global opportunistic pathogen. Clin. Microbiol. Rev. doi:10.1128/CMR.00019-11

Bush, K., 2018. Past and present perspectives on β-lactamases. Antimicrob. Agents Chemother. 62. doi:10.1128/aac.01076-18

Campana, E.H., Chuster, S.G., da Silva, I.R., Paschoal, R.P., Bonelli, R.R., Moreira, B.M., Picão, R.C., 2017. Modified Carba NP test for the detection of carbapenemase production in Gram-negative rods: optimized handling of multiple samples. Brazilian J. Microbiol. 48, 242–245. doi:10.1016/j.bjm.2016.09.015

CDC, 2013. Standard Operating Procedure for PulseNet PFGE of *Escherichia coli* O157:H7, *Escherichia coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri*.

Centers for Disease Control and Prevention, 2013. Antibiotic resistance threats in the United States, 2013. doi:CS239559-B

Chagas, T.P.G., Seki, L.M., Cury, J.C., Oliveira, J.A.L., Dávila, A.M.R., Silva, D.M., Asensi, M.D., 2011. Multiresistance, beta-lactamase-encoding genes and bacterial diversity in hospital wastewater in Rio de Janeiro, Brazil. J. Appl. Microbiol. 111, 572–81. doi:10.1111/j.1365-2672.2011.05072.x

Chun, J., Oren, A., Ventosa, A., Christensen, H., Arahal, D.R., da Costa, M.S., Rooney, A.P., Yi, H., Xu, X.W., De Meyer, S., Trujillo, M.E., 2018. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. Int. J. Syst. Evol. Microbiol. 68, 461–466. doi:10.1099/ijsem.0.002516

Clinical and Laboratory Standards Institute, 2015. Performance Standards for Antimicrobial Susceptibility Testing, 27th ed. ed. Wayne, PA, USA.

Correia, M., Boavida, F., Grosso, F., Salgado, M.J., Lito, L.M., Cristino, J.M., Mendo, S., Duarte, A., 2003. Molecular characterization of a new class 3 integron in Klebsiella pneumoniae. Antimicrob. Agents Chemother. 47, 2838–2843. doi:10.1128/AAC.47.9.2838-2843.2003

European Committee on Antimicrobial Susceptibility Testing, 2020. Breakpoint tables for interpretation of MICs and zone diameters.

European Environment Agency, 2017. Urban Waste Water Treatment. Off. J. Eur. Union 40–52.

European Environment Agency, 2016. Urban Waste Water Treatment maps [WWW Document].https://eea.maps.arcgis.com/apps/MapJournal/index.html?appid=e7e93bfd95ab44e28cae733b5a4ff54b &embed=true# (accessed 11.29.19).

Falagas, M.E., Kastoris, A.C., Vouloumanou, E.K., Rafailidis, P.I., Kapaskelis, A.M., Dimopoulos, G., 2009. Attributable mortality of *Stenotrophomonas maltophilia* infections: a systematic review of the literature. Future Microbiol. 4, 1103–1109. doi:10.2217/fmb.09.84

Fatta-kassinos, D., Kummerer, K., D. Dionysiou, D., 2015. Advanced Treatment

Technologies for Urban Wastewater. Springer, Cham. doi:10.1007/698_2015_359

Fernández, L., Hancock, R.E.W., 2012. Adaptive and mutational resistance: Role of porins and efflux pumps in drug resistance. Clin. Microbiol. Rev. 25, 661–681. doi:10.1128/CMR.00043-12

Gillings, M.R., 2014. Integrons: past, present, and future. Microbiol. Mol. Biol. Rev. 78, 257–77. doi:10.1128/MMBR.00056-13

Girlich, D., Naas, T., Nordmann, P., 2004. OXA-60, a chromosomal, inducible, and imipenem-hydrolyzing class D β-lactamase from *Ralstonia pickettii*. Antimicrob. Agents Chemother. 48, 4217–4225. doi:10.1128/AAC.48.11.4217-4225.2004

Girlich, D., Poirel, L., Szczepanowski, R., Schlüter, A., Nordmann, P., 2012. Carbapenem-hydrolyzing GES-5-encoding gene on different plasmid types recovered from a bacterial community in a sewage treatment plant. Appl. Environ. Microbiol. 78, 1292–1295. doi:10.1128/AEM.06841-11

Grundmann, H., Glasner, C., Albiger, B., Aanensen, D.M., Tomlinson, C.T., Andrasević, A.T., Cantón, R., Carmeli, Y., Friedrich, A.W., Giske, C.G., Glupczynski, Y., Gniadkowski, M., Livermore, D.M., Nordmann, P., Poirel, L., Rossolini, G.M., Seifert, H., Vatopoulos, A., Walsh, T., Woodford, N., Monnet, D.L., European Survey of Carbapenemase-Producing *Enterobacteriaceae* (EuSCAPE) Working Group, 2017. Occurrence of carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* in the European survey of carbapenemase-producing *Enterobacteriaceae* (EuSCAPE): a prospective, multinational study. Lancet Infect. Dis. 17, 153–163. doi:10.1016/S1473-3099(16)30257-2

Guardabassi, L., Lo Fo Wong, D.M.A., Dalsgaard, A., 2002. The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria. Water Res. 36, 1955–1964. doi:10.1016/S0043-1354(01)00429-8

Guo, M.-T., Yuan, Q.-B., Yang, J., 2013. Microbial selectivity of UV treatment on antibiotic-resistant heterotrophic bacteria in secondary effluents of a municipal wastewater treatment plant. Water Res. 47, 6388–94. doi:10.1016/j.watres.2013.08.012

Harmon, D.E., Miranda, O.A., McCarley, A., Eshaghian, M., Carlson, N., Ruiz, C., 2019. Prevalence and characterization of carbapenem-resistant bacteria in water bodies in the Los Angeles–Southern California area. Microbiologyopen

8, e00692. doi:10.1002/mbo3.692

Henriques, I., Moura, A., Alves, A., Saavedra, M.J., Correia, A., 2004. Molecular characterization of a carbapenem-hydrolyzing class A β-lactamase, SFC-1, from Serratia fonticola UTAD54. Antimicrob. Agents Chemother. 48, 2321–2324. doi:10.1128/AAC.48.6.2321-2324.2004

Henriques, I.S., Fonseca, F., Alves, A., Saavedra, M.J., Correia, A., 2006. Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from estuarine waters. Res. Microbiol. 157, 938–947. doi:10.1016/j.resmic.2006.09.003

Hrenovic, J., Ganjto, M., Goic-Barisic, I., 2017a. Carbapenem-resistant bacteria in a secondary wastewater treatment plant. Water SA 43, 186–191. doi:10.4314/wsa.v43i2.02

Hrenovic, J., Ivankovic, T., Ivekovic, D., Repec, S., Stipanicev, D., Ganjto, M., 2017b. The fate of carbapenem-resistant bacteria in a wastewater treatment plant. Water Res. 126, 232–239. doi:10.1016/j.watres.2017.09.007

Hu, Q., Zhang, X.X., Jia, S., Huang, K., Tang, J., Shi, P., Ye, L., Ren, H., 2016. Metagenomic insights into ultraviolet disinfection effects on antibiotic resistome in biologically treated wastewater. Water Res. 101, 309–317. doi:10.1016/j.watres.2016.05.092

Iovleva, A., Doi, Y., 2017. Carbapenem-Resistant *Enterobacteriaceae*. Clin. Lab. Med. 37, 303–315. doi:10.1016/j.cll.2017.01.005

Juhász, E., Krizsán, G., Lengyel, G., Grósz, G., Pongrácz, J., Kristóf, K., 2014. Infection and colonization by *Stenotrophomonas maltophilia*: Antimicrobial susceptibility and clinical background of strains isolated at a tertiary care centre in Hungary. Ann. Clin. Microbiol. Antimicrob. 13, 1–7. doi:10.1186/s12941-014-0058-9

Kelly, A.M., Mathema, B., Larson, E.L., 2017. Carbapenem-resistant *Enterobacteriaceae* in the community: a scoping review. Int. J. Antimicrob. Agents 50, 127–134. doi:10.1016/j.ijantimicag.2017.03.012

Kim, Y.J., Park, J.H., Seo, K.H., 2018. Presence of *Stenotrophomonas maltophilia* exhibiting high genetic similarity to clinical isolates in final effluents of pig farm wastewater treatment plants. Int. J. Hyg. Environ. Health 221, 300–307. doi:10.1016/j.ijheh.2017.12.002

Lamba, M., Ahammad, S.Z., 2017. Sewage treatment effluents in Delhi: A key contributor of β-lactam resistant bacteria and genes to the environment. Chemosphere 188, 249–256. doi:10.1016/j.chemosphere.2017.08.133

Lane, D., 1991. 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics, in: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley and Sons, New York, pp. 115–175.

Lee, I., Chalita, M., Ha, S.M., Na, S.I., Yoon, S.H., Chun, J., 2017. ContEst16S: An algorithm that identifies contaminated prokaryotic genomes using 16S RNA gene sequences. Int. J. Syst. Evol. Microbiol. 67, 2053–2057. doi:10.1099/ijsem.0.001872

Magiorakos, A.P., Burns, K., Rodríguez Baño, J., Borg, M., Daikos, G., Dumpis, U., Lucet, J.C., Moro, M.L., Tacconelli, E., Simonsen, G.S., Szilágyi, E., Voss, A., Weber, J.T., 2017. Infection prevention and control measures and tools for the prevention of entry of carbapenem-resistant *Enterobacteriaceae* into healthcare settings: Guidance from the European Centre for Disease Prevention and Control. Antimicrob. Resist. Infect. Control 6, 113. doi:10.1186/s13756-017-0259-z

Manageiro, V., Ferreira, E., Almeida, J., Barbosa, S., Simões, C., Bonomo, R.A., Caniça, M., Castro, A.P., Lopes, P., Fonseca, F., Vieira, S., Guimarães, M.A., Ribeiro, J., Oliveira, H., Pinto, M., Diogo, J., Jesus, A., Sancho, L., Rodrigues, M., Vaz, T., Afonso, T., 2015. Predominance of KPC-3 in a survey for carbapenemase-producing *Enterobacteriaceae* in Portugal. Antimicrob. Agents Chemother. 59, 3588–3592. doi:10.1128/AAC.05065-14

Manageiro, V., Ferreira, E., Caniça, M., Manaia, C.M., 2014a. GES-5 among the β-lactamases detected in ubiquitous bacteria isolated from aquatic environment samples. FEMS Microbiol. Lett. 351, 64–69. doi:10.1111/1574-6968.12340

Manageiro, V., Ferreira, E., Pinto, M., Caniça, M., 2014b. First description of OXA-48 carbapenemase harbored by *Escherichia coli* and *Enterobacter cloacae* in a single patient, in Portugal. Antimicrob. Agents Chemother. 1–6. doi:10.1128/AAC.02961-14

Manageiro, V., Romão, R., Moura, I.B., Sampaio, D.A., Vieira, L., Ferreira, E., Caniça, M., 2018. Molecular epidemiology and risk factors of carbapenemase-producing *Enterobacteriaceae* isolates in Portuguese

hospitals: Results from European survey on carbapenemase-producing *Enterobacteriaceae* (EuSCAPE). Front. Microbiol. 9, 2834. doi:10.3389/fmicb.2018.02834

Manaia, C.M., Macedo, G., Fatta-Kassinos, D., Nunes, O.C., 2016. Antibiotic resistance in urban aquatic environments: can it be controlled? Appl. Microbiol. Biotechnol. 100, 1543–1557. doi:10.1007/s00253-015-7202-0

Marano, R.B.M., Fernandes, T., Manaia, C.M., Nunes, O., Morrison, D., Berendonk, T.U., Kreuzinger, N., Telson, T., Corno, G., Fatta-Kassinos, D., Merlin, C., Topp, E., Jurkevitch, E., Henn, L., Scott, A., Heß, S., Slipko, K., Laht, M., Kisand, V., Di Cesare, A., Karaolia, P., Michael, S.G., Petre, A.L., Rosal, R., Pruden, A., Riquelme, V., Agüera, A., Esteban, B., Luczkiewicz, A., Kalinowska, A., Leonard, A., Gaze, W.H., Adegoke, A.A., Stenstrom, T.A., Pollice, A., Salerno, C., Schwermer, C.U., Krzeminski, P., Guilloteau, H., Donner, E., Drigo, B., Libralato, G., Guida, M., Bürgmann, H., Beck, K., Garelick, H., Tacão, M., Henriques, I., Martínez-Alcalá, I., Guillén-Navarro, J.M., Popowska, M., Piotrowska, M., Quintela-Baluja, M., Bunce, J.T., Polo-López, M.I., Nahim–Granados, S., Pons, M.N., Milakovic, M., Udikovic-Kolic, N., Ory, J., Ousmane, T., Caballero, P., Oliver, A., Rodriguez-Mozaz, S., Balcazar, J.L., Jäger, T., Schwartz, T., Yang, Y., Zou, S., Lee, Y., Yoon, Y., Herzog, B., Mayrhofer, H., Prakash, O., Nimonkar, Y., Heath, E., Baraniak, A., Abreu-Silva, J., Choudhury, M., Munoz, L.P., Krizanovic, S., Brunetti, G., Maile-Moskowitz, A., Brown, C., Cytryn, E., 2020. A global multinational survey of cefotaxime-resistant coliforms in urban wastewater treatment plants. Environ. Int. 144, 106035. doi:10.1016/j.envint.2020.106035

McArthur, A.G., Waglechner, N., Nizam, F., Yan, A., Azad, M.A., Baylay, A.J., Bhullar, K., Canova, M.J., De Pascale, G., Ejim, L., Kalan, L., King, A.M., Koteva, K., Morar, M., Mulvey, M.R., O'Brien, J.S., Pawlowski, A.C., Piddock, L.J.V., Spanogiannopoulos, P., Sutherland, A.D., Tang, I., Taylor, P.L., Thaker, M., Wang, W., Yan, M., Yu, T., Wright, G.D., 2013. The comprehensive antibiotic resistance database. Antimicrob. Agents Chemother. 57, 3348–3357. doi:10.1128/AAC.00419-13

McKinney, C.W., Pruden, A., 2012. Ultraviolet disinfection of antibiotic resistant bacteria and their antibiotic resistance genes in water and wastewater. Environ. Sci. Technol. 46, 13393–13400. doi:10.1021/es303652q

Meier-Kolthoff, J.P., Auch, A.F., Klenk, H.P., Göker, M., 2013. Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatics 14, 60. doi:10.1186/1471-2105-14-60

Meier-Kolthoff, J.P., Klenk, H.P., Göker, M., 2014. Taxonomic use of DNA G+C content and DNA-DNA hybridization in the genomic age. Int. J. Syst. Evol. Microbiol. 64, 352–356. doi:10.1099/ijs.0.056994-0

Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review. Water Res. 47, 957–995. doi:10.1016/j.watres.2012.11.027

Ministério do Ambiente e Transição Energética, 2019. Agência Portuguesa do Ambiente (APA).

Mir-Tutusaus, J.A., Parladé, E., Villagrasa, M., Barceló, D., Rodríguez-Mozaz, S., Martínez-Alonso, M., Gaju, N., Sarrà, M., Caminal, G., 2019. Long-term continuous treatment of non-sterile real hospital wastewater by Trametes versicolor. J. Biol. Eng. 13, 47. doi:10.1186/s13036-019-0179-y

Moura, A., Araújo, S., Alves, M.S., Henriques, I., Pereira, A., Correia, A.C.M.A.C.M., 2014. The contribution of *Escherichia coli* from human and animal sources to the integron gene pool in coastal waters. Front. Microbiol. 5, 1–15. doi:10.3389/fmicb.2014.00419

Moura, A., Henriques, I., Ribeiro, R., Correia, A., 2007. Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. J. Antimicrob. Chemother. 60, 1243–1250. doi:10.1093/jac/dkm340

Moura, A., Henriques, I., Smalla, K., Correia, A., 2010. Wastewater bacterial communities bring together broad-host range plasmids, integrons and a wide diversity of uncharacterized gene cassettes. Res. Microbiol. 161, 58–66. doi:10.1016/j.resmic.2009.11.004

Munir, M., Wong, K., Xagoraraki, I., 2011. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. Water Res. 45, 681–693. doi:10.1016/j.watres.2010.08.033

Naas, T., Poirel, L., Nordmann, P., 2008. Minor extended-spectrum β-lactamases. Clin. Microbiol. Infect. 14, 42–52. doi:10.1111/j.1469-0691.2007.01861.x

Nicolas-Chanoine, M.H., Mayer, N., Guyot, K., Dumont, E., Pagès, J.M., 2018. Interplay between membrane permeability and enzymatic barrier leads to antibiotic-dependent resistance in *Klebsiella pneumoniae*. Front. Microbiol. 9, 1422. doi:10.3389/fmicb.2018.01422

Nordmann, P., Poirel, L., 2014. The difficult-to-control spread of carbapenemase producers among *Enterobacteriaceae* worldwide. Clin. Microbiol. Infect. 20, 821–830. doi:10.1111/1469-0691.12719

Oyeka, M., Antony, S., 2017. *Citrobacter braakii* Bacteremia: Case Report and Review of the Literature. Infect. Disord. - Drug Targets 17, 59–63. doi:10.2174/1871526516666161005155847

Pancotto, L.R., Nodari, C.S., Rozales, F.P., Soldi, T., Siqueira, C.G., Freitas, A.L., Barth, A.L., 2018. Performance of rapid tests for carbapenemase detection among Brazilian *Enterobacteriaceae* isolates. Brazilian J. Microbiol. 49, 914–918. doi:10.1016/j.bjm.2018.07.002

Papagiannitsis, C.C., Dolejska, M., Izdebski, R., Dobiasova, H., Studentova, V., Esteves, F.J., Derde, L.P.G., Bonten, M.J.M., Hrabák, J., Gniadkowski, M., 2015. Characterization of pKP-M1144, a Novel ColE1-like plasmid encoding IMP-8, GES-5, and BEL-1 β-lactamases, from a *Klebsiella pneumoniae* sequence type 252 isolate. Antimicrob. Agents Chemother. 59, 5065–5068. doi:10.1128/AAC.00937-15

Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., Tyson, G.W., 2015. CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 25, 1043–1055. doi:10.1101/gr.186072.114

Pei, M., Zhang, B., He, Y., Su, J., Gin, K., Lev, O., Shen, G., Hu, S., 2019. State of the art of tertiary treatment technologies for controlling antibiotic resistance in wastewater treatment plants. Environ. Int. 131, 105026. doi:10.1016/j.envint.2019.105026

Picão, R.C., Cardoso, J.P., Campana, E.H., Nicoletti, A.G., Petrolini, F.V.B., Assis, D.M., Juliano, L., Gales, A.C., 2013. The route of antimicrobial resistance from the hospital effluent to the environment: Focus on the occurrence of KPC-producing *Aeromonas* spp. and *Enterobacteriaceae* in sewage. Diagn. Microbiol. Infect. Dis. 76, 80–85. doi:10.1016/j.diagmicrobio.2013.02.001

Pither, M.D., McClean, S., Silipo, A., Molinaro, A., Di Lorenzo, F., 2020. A chronic strain of the cystic fibrosis pathogen *Pandoraea pulmonicola* expresses a heterogenous hypo-acylated lipid A. Glycoconj. J. 1–10. doi:10.1007/s10719-020-09954-8

Poirel, L., Brinas, L., Verlinde, A., Ide, L., Nordmann, P., 2005. BEL-1, a novel clavulanic acid-inhibited extended-spectrum β-lactamase, and the class 1

integron In120 in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 49, 3743–3748. doi:10.1128/AAC.49.9.3743-3748.2005

Poirel, L., Carattoli, A., Bernabeu, S., Bruderer, T., Frei, R., Nordmann, P., 2010. A novel IncQ plasmid type harbouring a class 3 integron from *Escherichia coli*. J. Antimicrob. Chemother. 65, 1594–1598. doi:10.1093/jac/dkq166

Poirel, L., Carrër, A., Pitout, J.D., Nordmann, P., 2009. Integron mobilization unit as a source of mobility of antibiotic resistance genes. Antimicrob. Agents Chemother. 53, 2492–2498. doi:10.1128/AAC.00033-09

Poirel, L., Héritier, C., Podglajen, I., Sougakoff, W., Gutmann, L., Nordmann, P., 2003. Emergence in *Klebsiella pneumoniae* of a chromosome-encoded SHV β-lactamase that compromises the efficacy of imipenem. Antimicrob. Agents Chemother. 47, 755–758. doi:10.1128/AAC.47.2.755-758.2003

Potter, R.F., D'Souza, A.W., Dantas, G., 2016. The rapid spread of carbapenem-resistant *Enterobacteriaceae*. Drug Resist. Updat. 29, 30–46. doi:10.1016/j.drup.2016.09.002

Programa de Prevenção e Controlo de Infeções e de Resistência aos Antimicrobianos, 2017. RECOMENDAÇÃO - Prevenção da Transmissão de enterobacteriáceas resistentes aos carbapenemos em hospitais de cuidados de agudos.

Pruden, A., 2014. Balancing water sustainability and public health goals in the face of growing concerns about antibiotic resistance. Environ. Sci. Technol. 48, 5–14. doi:10.1021/es403883p

Queenan, A.M., Bush, K., 2007. Carbapenemases: The versatile β-lactamases. Clin. Microbiol. Rev. 20, 440–458. doi:10.1128/CMR.00001-07

R Development Core Team, 2008. R: A language and environment for statistical computing. R Foundation for statistical Computing.

Reinke, R.A., Quach-Cu, J., Allison, N., Lynch, B., Crisostomo, C., Padilla, M., 2020. A method to quantify viable carbapenem resistant Gram-negative bacteria in treated and untreated wastewater. J. Microbiol. Methods 179, 106070. doi:10.1016/j.mimet.2020.106070

Richter, M., Rosselló-Móra, R., Oliver Glöckner, F., Peplies, J., 2016. JSpeciesWS: A web server for prokaryotic species circumscription based on pairwise genome comparison. Bioinformatics 32, 929–931.

doi:10.1093/bioinformatics/btv681

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. 447, 345–360. doi:10.1016/j.scitotenv.2013.01.032

Runcharoen, C., Raven, K.E., Reuter, S., Kallonen, T., Paksanont, S., Thammachote, J., Anun, S., Blane, B., Parkhill, J., Peacock, S.J., Chantratita, N., 2017. Whole genome sequencing of ESBL-producing *Escherichia coli* isolated from patients, farm waste and canals in Thailand. Genome Med. 9, 81. doi:10.1186/s13073-017-0471-8

Sakkas, H., Bozidis, P., Ilia, A., Mpekoulis, G., Papadopoulou, C., 2019. Antimicrobial resistance in bacterial pathogens and detection of carbapenemases in *Klebsiella pneumoniae* isolates from hospital wastewater. Antibiotics 8. doi:10.3390/antibiotics8030085

Schlüter, A., Szczepanowski, R., Pühler, A., Top, E.M., 2007. Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. FEMS Microbiol. Rev. 31, 449–477. doi:10.1111/j.1574-6976.2007.00074.x

Schneider, I., Queenan, A.M., Bauernfeind, A., 2006. Novel carbapenem-hydrolyzing oxacillinase OXA-62 from *Pandoraea pnomenusa*. Antimicrob. Agents Chemother. 50, 1330–1335. doi:10.1128/AAC.50.4.1330-1335.2006

Serna-Galvis, E.A., Salazar-Ospina, L., Jiménez, J.N., Pino, N.J., Torres-Palma, R.A., 2018. Elimination of carbapenem resistant *Klebsiella pneumoniae* in water by UV-C, UV-C/persulfate and UV-C/H2O2. Evaluation of response to antibiotic, residual effect of the processes and removal of resistance gene. J. Environ. Chem. Eng. doi:10.1016/j.jece.2018.02.004

Silva, I., Tacão, M., Tavares, R.D.S., Miranda, R., Araújo, S., Manaia, C.M., Henriques, I., 2018. Fate of cefotaxime-resistant *Enterobacteriaceae* and ESBL-producers over a full-scale wastewater treatment process with UV disinfection. Sci. Total Environ. 639, 1028–1037. doi:10.1016/j.scitotenv.2018.05.229

Tacão, M., Araújo, S., Vendas, M., Alves, A., Henriques, I., 2018. *Shewanella* species as the origin of $bla_{\text{OXA-48}}$ genes: insights into gene diversity, associated phenotypes and possible transfer mechanisms. Int. J. Antimicrob. Agents 51, 340–348. doi:10.1016/j.ijantimicag.2017.05.014

Tacão, M., Correia, A.C.M., Henriques, I.S., 2015. Low prevalence of carbapenem-resistant bacteria in river water: resistance is mostly related to intrinsic mechanisms. Microb. Drug Resist. 21, 497–506. doi:10.1089/mdr.2015.0072

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Teixeira, P., Tacão, M., Pureza, L., Gonçalves, J., Silva, A., Cruz-Schneider, M.P., Henriques, I., 2020. Occurrence of carbapenemase-producing *Enterobacteriaceae* in a Portuguese river: *bla*$_{NDM}$, *bla*$_{KPC}$ and *bla*$_{GES}$ among the detected genes. Environ. Pollut. 260. doi:10.1016/j.envpol.2020.113913

Tijet, N., Boyd, D., Patel, S.N., Mulvey, M.R., Melano, R.G., 2013. Evaluation of the carba NP test for rapid detection of carbapenemase-producing *Enterobacteriaceae* and *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 57, 4578–4580. doi:10.1128/AAC.00878-13

Tzouvelekis, L.S., Markogiannakis, A., Psichogiou, M., Tassios, P.T., Daikos, G.L., 2012. Carbapenemases in *Klebsiella pneumoniae* and other *Enterobacteriaceae*: An evolving crisis of global dimensions. Clin. Microbiol. Rev. 25, 682–707. doi:10.1128/CMR.05035-11

Vourli, S., Giakkoupi, P., Miriagou, V., Tzelepi, E., Vatopoulos, A.C., Tzouvelekis, L.S., 2004. Novel GES/IBC extended-spectrum β-lactamase variants with carbapenemase activity in clinical enterobacteria. FEMS Microbiol. Lett. 234, 209–213. doi:10.1016/j.femsle.2004.03.028

Vubil, D., Figueiredo, R., Reis, T., Canha, C., Boaventura, L., Da Silva, G.J., 2017. Outbreak of KPC-3-producing ST15 and ST348 *Klebsiella pneumoniae* in a Portuguese hospital. Epidemiol. Infect. 145, 595–599. doi:10.1017/S0950268816002442

Wang, Y.L., Scipione, M.R., Dubrovskaya, Y., Papadopoulos, J., 2014. Monotherapy with fluoroquinolone or trimethoprim-sulfamethoxazole for treatment of *Stenotrophomonas maltophilia* infections. Antimicrob. Agents Chemother. 58, 176–182. doi:10.1128/AAC.01751-13

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

doi:10.1016/j.jhin.2016.03.007

WHO, 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. doi:10.1016/S1473-3099(09)70222-1

Wickham, H., 2016. ggplot2: Elegant graphics for data analysis. Springer-Verlag, New York.

Wickham, H., François, R., Henry, L., Müller, K., 2018. dplyr: A grammar of data manipulation., R package. ed.

Xu, T., Wang, J., Ying, Jianchao, Zhu, T., Liu, Y., Xu, L., Li, Pingping, Li, Peizhen, Ying, Jun, Li, K., Yi, H., Lu, J., Hu, Y., Zhou, T., Bao, Q., 2018. Characterisation of a class 1 integron associated with the formation of quadruple *bla*$_{GES-5}$ cassettes from an IncP-1*β* group group plasmid in *Pseudomonas aeruginosa*. Int. J. Antimicrob. Agents 52, 485–491. doi:10.1016/j.ijantimicag.2018.07.002

Yang, F., Mao, D., Zhou, H., Luo, Y., 2016. Prevalence and fate of carbapenemase genes in a wastewater treatment plant in northern China. PLoS One 11, e0156383. doi:10.1371/journal.pone.0156383

Zhang, L., Ma, X., Luo, L., Hu, N., Duan, J., Tang, Z., Zhong, R., Li, Y., 2020. The prevalence and characterization of extended-spectrum β-lactamase-and carbapenemase-producing bacteria from hospital sewage, treated effluents and receiving rivers. Int. J. Environ. Res. Public Health 17, 1183. doi:10.3390/ijerph17041183

Zhang, L.I., Xian-Zhi, L.I., Poole, K., 2000. Multiple antibiotic resistance in *Stenotrophomonas maltophilia*: Involvement of a multidrug efflux system. Antimicrob. Agents Chemother. 44, 287–293. doi:10.1128/AAC.44.2.287-293.2000

III. FINAL CONSIDERATIONS

Antimicrobial-resistant microbes can be recovered from humans, animals, food, and the environment. There are no geographical boundaries or species borders for their arise and dispersion. They can spread between humans and other animals, including food animals, and from person to person. Deficient infection control and sanitary conditions, as well as inappropriate food-handling and water management instigate the endless spread of antimicrobial resistance. To tackle this rising threat a holistic, multisectoral, and transdisciplinary tactic is required – the so-called One Health approach. Acknowledging natural environments' role in the antibiotic resistance (AR) emergence and dissemination is imperative to understand and mitigate this problem and to encourage innovation in the area of antibiotics, meeting global needs.

Preservation of the natural balance within and between ecosystems is crucial. Parallelly, surveillance of environmental settings is fundamental and still remarkably limited. Measures should be taken to contain antimicrobial resistance spread in ecosystems and, for that, further studies are needed to define which bacteria, which resistance determinants, and which transfer mechanisms exist in a given ecosystem. In this context, aquatic environments are one of the most critical areas to lean into, even more as they are frequently impacted by anthropogenic pressure. It is particularly important to address aquatic systems used by men directly, either for drinking, leisure, or for irrigation purposes, to name a few. However, addressing those aquatic systems that function as links between pristine environments and the civilization settings – as much as it can be possibly tackled – is highly important as well.

Aquatic settings are reservoirs of indigenous resistant bacteria and genes, and are receptors of diverse contaminants. Furthermore, ubiquitous bacteria that thrive in aquatic environments carrying clinically relevant resistance determinants are a realistic scenario for the last couple of decades. Hence aquatic environments are likely prone to microbial promiscuity due to their natural characteristics, ideal for bacterial prosperity; which altogether, constitute the ultimate conditions to occur transmission of genes between native and non-native bacteria, thus contributing to the exacerbation of this problematic.

Understanding the resistome and mobilome of these environmental systems is essential to further elucidate the role of human activities in the dissemination

and persistence of AR, particularly in what concerns antibiotics critically relevant in healthcare systems, e.g. used to treat severe infections caused by Gram-negative bacteria. Hence, several chapters of this study were focused on resistance towards last-resort antibiotics, particularly carbapenems, critically important to human health that are used as final treatment options for dealing with infections caused by multiresistant strains.

Specially since the last decade, awareness from various organizations with reports dedicated to carbapenem resistance has significantly risen (CDC, 2013; ECDC, 2019; WHO, 2017). In particular, the wholesome – and specifics – knowledge in aquatic environments regarding AR, and more specifically carbapenem resistance, is still limited, and in this line of investigation, additional knowledge can never be underestimated.

Aligned with the latest reports, our research in the majority of the chapters has emphasized particularly the family *Enterobacteriaceae* that is abidingly mentioned therein. This group of bacteria, which includes clinically relevant human and animal pathogenic species, are implicated in infection cases worldwide. Their emergence allegedly coincides with the emergence of mammalian organisms, with which they are closely adapted, namely to the gut of mammals. However, their high genome plasticity and flexible gene pool turn them highly adaptable and makes them relevant to be included in this work.

Through this particular perspective, we managed to assess the aims proposed, and by this way, confirm the hypotheses outlined.

Hypothesis 1: Environmental bacteria, namely from aquatic environments, are the progenitors of antibiotic-resistant genes (ARGs) relevant in clinical settings.

This hypothesis was addressed in the first two chapters. In order to test this premise, we assessed the role of bacteria of the genus *Shewanella* as the natural reservoir of two families of ARGs.

This work allowed to reinforce the putative origin in this environmental genus of the gene families *bla*$_{\text{OXA-48}}$-like and *qnrA*, (Chapter 1 and Chapter 2, respectively).

bla$_{OXA-48}$-like genes presence or absence was species-related (Chapter 1), and its location was confirmed to be chromosomal. Besides the already established progenitor species – *S. xiamenensis* (Potron et al., 2011) –, several other *Shewanella* spp. carried a putative *bla*$_{OXA-48}$-like, namely *S. baltica* and *S. putrefaciens*. The same happened in the case of *qnrA*-like genes (Chapter 2), being that in addition to the beforehand species considered to be the putative origin of the gene – *S. algae* (Poirel et al., 2005)–, other *Shewanella* spp. were found as *qnrA*-like-carrying, including *S. indica* and *S. haliotis*. These are shreds of evidence that consolidate these AR determinants as intrinsic to at least some members of the *Shewanella* genus. The determinants may have once existed in all shewanellae members and were lost in some species, or species divergence from a common ancestor may have occurred before the gene(s) emergence.

The occurrence of OXA-48-like-producing shewanellae, particularly *S. xiamenensis*, *S. algae* and *S. haliotis*, and the two latter producing a QnrA-like enzyme, is of concern, representing a potential human health risk, as these bacteria have been increasingly identified as opportunistic human pathogens (Almuzara et al., 2017; Janda, 2014; Poovorawan et al., 2013; Tsai et al., 2008; Zong, 2011). Even though these genes are intrinsic, with low risk of being transferred to another host, it is possible to infer, from their similar hydrolytic profile to the ones described in non-shewanellae bacteria, that in the eventuality of causing an infection it would compromise the treatment with a carbapenem antibiotic.

Nonetheless, we obtained a set of evidences of genetic determinants allocated to mobilization function and potential gene exchange. Genetic determinants that mediate gene flow, like insertion sequences (ISs), were found to be present in most of the *bla*$_{OXA-48}$-carrying *Shewanella* isolates (IS*1999*) and also on a few genomes (available at the time). Although not being detected within the same genetic context as the resistance genes, its detection in the majority of the *Shewanella* isolates and frequent occurrence in *Enterobacteriaceae* (Aubert et al., 2006; Carrër et al., 2010; Poirel et al., 2012) suggests that this IS may play a role in the mobilization mechanism and transfer process of this gene from the original reservoir to other hosts. Moreover, the low prevalence detected in genomes might be an indicator that its acquisition might be dependent on the adaptation to a certain environment. Further investigation should be conducted, as the

number of genomes available in databases increased considerably since this study (Bradley et al., 2019), and this investigation should include strains from other environments, namely from clinical settings, to clarify the role of this IS. Based on the promiscuous conditions that aquatic environments provide we can speculate that the transmission of *bla*$_{OXA-48}$-like genes to other hosts may be more prone to occur in these settings.

The results of the first two Chapters contribute to emphasize the already growing idea that the origin of some clinically important ARGs is in environmental settings. Hence, it becomes clear how crucial it is to address water compartments as key points of further and deeper investigation.

Hypothesis 2: Anthropogenic activities potentiate the dissemination of bacterial resistance in environmental ecosystems.

Like other ecosystems but perhaps more, as the water cycle mediates so many processes of the living world, aquatic environments are continuously affected by human actions, including discharges of antibiotics, antibiotic-resistant bacteria (ARB), ARGs, and other contaminants that facilitate AR selection. Water is an important vehicle between natural compartments, animals, and humans, facilitating the transmission to – and between – animals and humans.

For the second part of the work, it was hypothesized that human activities impact the environment by promoting the spread of bacterial resistance. To confirm this we assessed the role of wastewater as a contributor to the contamination of aquatic systems, and the role of groundwater used for irrigation in the contamination of fresh produce usually eaten raw.

In Chapter 3, a collection of *E. coli* strains, retrieved from irrigation water and vegetables of household origin, was characterized for its antibiotic resistance, virulence attributes, and the occurrence of mobile genetic determinants. In Chapter 4, two MDR *E. coli* isolates from the latter chapter, were selected for whole-genome sequencing and *in silico* analysis.

Groundwater can be affected in diverse ways, not only by natural phenomena (e.g. surface runoffs) but also, and most impacting, by human compelled

activities, namely agriculture practices as fertilizers or pesticides use (Hansen et al., 2011; Malki et al., 2017; Virgílio Cruz et al., 2013). In the majority of small domestic farms, groundwater is commonly used in the production of fresh crops. The lack of established monitoring programs to assess its microbiological quality turns considerably difficult to monitor both groundwater and the fresh produce. Evidence was found in this work that contaminated irrigation water constitutes a potential route for *E. coli* to enter the food chain by raw-eaten vegetables.

Strains found in both sources (lettuce and water), including clonal groups, were mostly from phylogenetic groups A and B1, that have been frequently associated with DEC strains. Herein ETEC and EIEC strains were detected integrating phylogroup B1. The STs detected in these isolates, namely ST48, are frequently associated with clinically relevant ARGs and infections documented worldwide (Hassan et al., 2020; Leverstein-van Hall et al., 2011; Liu et al., 2019; Madec et al., 2016; Sato et al., 2017; Smet et al., 2010). The fact that the same strains were found in water and vegetables, and clonal groups were detected among several household productions, is of concern suggesting cross-contamination, most probably through the groundwater contamination.

In this study *E. coli* strains were frequently MDR and, in some cases, their acquired resistance determinants were proven to be transferable to a different host. Moreover, all the integrons were detected in MDR isolates, and possessed gene cassette arrays previously reported in several bacteria, including *E. coli*, from different environmental settings (Integrall Database - Moura et al., 2009). These results highlight these platforms' role in the dissemination of antibiotic resistance traits and co-selection. Regarding the potential of these strains to be co-selected by antibiotic and metal-resistance (Dickinson et al., 2019; Henriques et al., 2016), whole-genome sequencing of two *E. coli* strains (Chapter 4) allowed the detection of genes encoding for metals tolerance.

Furthermore, conjugative plasmids were found among several clonal groups. The plasmid replicons identified here are commonly associated to bacteria causing human infections (Ahmad et al., 2019; Chah et al., 2010; Jahanbakhsh et al., 2016; Shahada et al., 2013; Xu et al., 2019). An example is the replicon IncFIC which is extremely rare to find among environmental isolates (Balbin et al., 2020;

Zou et al., 2020) and was present in a clonal group widespread among different producers.

The whole-genome sequencing of two isolates that presented a MDR phenotype (Chapter 4) made it possible to withdraw several other conclusions. These two strains had several flash traits, including the fact that although their integrons could be detected by molecular screening, it was not possible to determine by PCR their gene cassettes arrangements. By whole-genome sequence analysis, class 1 integron arrays were identified, being highly similar to an integron identified in a plasmid detected in *E. coli* (unpublished; accession no. CP034786). These isolates presented MDR traits (to streptomycin, tetracycline, and sulfamethoxazole/trimethoprim), and curiously the plasmid transferred to other host suggested the presence of other ARGs that had not been detected by PCR screening. By genome analysis, it was possible to confirm the presence of some of those genes responsible for the phenotypes determined in Chapter 3. Also, this strategy allowed to identify a wide array of virulence factors and genes related to metal tolerance.

Moreover, elements responsible for mobilization, like IS-like transposases were also detected as well as a phage integrase-encoding gene. Additionally, this *in silico* analysis allowed to determine the serotype of the Y15V.22 strain. To our knowledge, these strains represent the first MDR *E. coli* O8:H9-ST48 reported in Portugal. Nevertheless, these results are in agreement with other studies where strains with these characteristics were reported carrying clinically relevant ARGs in humans and food sources (Liu et al., 2017; Luo et al., 2017). This denotes the possibility of this clone playing a role in the dissemination of AR between the environment and humans, as it was obtained from water and vegetables.

In the last Chapter (5), a collection of carbapenem-resistant Gram-negative bacteria was characterized, and carbapenem-resistant *Enterobacteriaceae* (CRE) strains obtained from raw water were characterized by whole-genome sequencing.

The evaluation of antibiotic resistance levels in residual waters, particularly the presence of resistance traits to antibiotics used as last-line drugs as carbapenems, is crucial to combat the spread of bacterial resistance. This knowledge may help

to mitigate a problem that can be anticipated by observing the increasing levels of resistance to this specific group of antimicrobials in clinical settings.

Disinfection by UV-C radiation is one of the existing tertiary disinfection methods which are applied to residual waters of all sources to eliminate ARB, ARGs, and other potential risk traits, so that the final effluent can be considered safe to release in the environment. Although after secondary treatment an higher prevalence of meropenem-resistant bacteria was observed, suggesting a positive selection – which is in alignment with previous studies (Hrenovic et al., 2017) –, UV-Cs proved to be effective in decreasing the abundance of total bacteria and the prevalence of carbapenem-resistant bacteria (CRB). However, an increase of CRB prevalence was detected after a period of incubation of the final effluent in the dark. This result can be explained by the fact that the majority of the bacteria detected after the UV-C treatment belonged to species with intrinsic resistance mechanisms such as *Stenotrophomonas maltophilia* that may reacquire capacity to replicate or regrow. As this step was intentioned to mimic what happens in reality, since WWTPs have post-treatment aquifers (with minimum or no light) in order to avoid photoreactivation of the injured bacteria, the resulted water quality is of great concern.

CRE detection only in raw water further suggests the efficacy of this treatment. Although rare, there have been reports of CRE detection in final effluents even when advanced treatments are applied (Lamba and Ahammad, 2017; Yang et al., 2016), and its increase cannot be discarded as a possibility in the near future, as the levels of CRB in clinical settings, and consequently in the community, are also increasing. These findings suggest, in accordance with previous reports (Lamba and Ahammad, 2017; Yang et al., 2016), that under certain conditions these bacteria, namely light conditions, may reactivate or repair their resistance mechanisms, surviving the disinfection processes and, ultimately, be released into the environment. By these means, the occurrence of CRE in residual waters is by itself a warning sign that must be considered in the near future.

CRB detected in this study affiliated to pathogenic species for humans, such as *Citrobacter* spp.. The genera found in raw water, such as *Citrobacter*, but also *Enterobacter*, *Leclercia* and *Lelliottia* species, is a predictable discovery as they

are members of the *Enterobacteriaceae* family regarded as natural colonizers of the normal gut flora of animals and humans and are also found in a variety of environments (Kämpfer et al., 2018; Ribeiro et al., 2017; Sun et al., 2019). Regarding the total bacterial diversity carrying carbapenemases in these environments, by the *Lelliottia* sp. isolate found in the raw water which most likely is a novel specie, it is perceptible that much clarifies is still to be discovered. Additionally, all the CRE isolates possessed the acquired carbapenemase gene bla_{GES-5}. This was a surprising finding since this gene is still rarely reported in clinical settings in Portugal even if, in line with our findings, the GES-5 enzyme had been already detected in aquatic systems in Portugal (Manageiro et al., 2014; Teixeira et al., 2020). The *in silico* analysis of the genetic context of this carbapenemase gene allowed to understand that this gene is associated with class 1 and 3 integrons. In several cases integrons appeared associated to mobilizable plasmids underlining again the importance of these platforms in the mobilization and gathering of several distinct ARGs, contributing to the spread of MDR phenotypes. Other bacteria were identified in the final effluent such as *Stenotrophomonas maltophilia*, which have been described as emergent opportunistic pathogens responsible for severe MDR infections in humans (Adegoke et al., 2017; Kim et al., 2018).

Methodological considerations

Our findings were based on culture-dependent methods, which is the standard in clinical resistance research. Although this approach is known to underestimate the composition of the environmental microbial resistome, the data gathered in this investigation proved this approach to be very useful in achieving the proposed aims.

These methods allow to obtain bacterial isolates and thus to obtain phenotypic and genotypic data, which in this case was essential. Some examples of the advantages encountered are: the use of selective media that proved to be very important to focus in the analysis on bacterial groups of special interest in terms of human health, such as *E. coli* for which the HiCrome *E. coli* agar B

medium was used (Chapter 3); also, the isolates obtained made it possible to perform mating assays, which allowed to confirm the dissemination potential of some of the mobile genetic elements identified (Chapter 1 and 3); we were able to assess the antibiotic susceptibility patterns of the bacterial isolates (all Chapters); and we identified a carbapenem-resistant isolate that most likely represents a novel species, which may be relevant to human health (Chapter 5).

A genomic approach was also used as a complementary tool to evaluate antibiotic resistance determinants in different contexts, and to assess the putative risk of the obtained isolates to human health. The methodology was applied to isolates obtained during this study and to deposited genomes. This approach proved to be very useful to achieve the objectives, allowing a more global view of the diversity of genes present in each species and allowing the identification of new variants of these genes (Chapter 1 and 2).

Finally, whole genome sequencing represented an added value to the work, as it allowed a more reliable and detailed identification of the bacteria analyzed, a detailed analysis of the resistome and mobilome, as well as in the genes context assessment. This methodology allowed, for example, to clarify the structure of integrons that were not possible to clarify by more "traditional" methodologies based on PCR and Sanger sequencing (Chapter 4). Also, it was possible to detect genes encoding metal tolerance, which were not assessed by other methodologies.

Concluding remarks

The results obtained in this work reinforce the important role that aquatic systems have in the emergence and spread of AR, highlighting the necessity for strategies of water quality improvement. *Shewanella* spp. were proven to be reservoirs of $bla_{\text{OXA-48}}$- and *qnrA*-like genes. As seen in the third chapter one thing that could make a difference in the household context was to regularly monitor the quality of the groundwater. Until this date, the microbiological quality of both private water wells and vegetables produced in small domestic farms has been

poorly addressed – if at all –, in Portugal. This work contributed to overcome this lack, identifying water-associated risk factors and demonstrating how water bodies can easily be conductive lines in the continuous spread of AR. Advanced wastewater treatments, as solutions to eliminate pollutants of different origins, although efficient in removing bacteria, should be further assessed as these processes may select for bacteria intrinsically resistant to antibiotics, some of which may emerge as opportunistic pathogens. Moreover, the mere fact of encountering CRE in raw water is of concern as a greater risk for human health for which a close surveillance is recommended.

In our understanding only an integrated vision can provide elements to assess the risk of the spread of AR in water bodies. Analyses of different environmental compartments, focusing on the sources, reservoirs and possible transmission routes to humans, stands as an useful strategy to accomplish a much-demanded change and solutions for this urgent health issue.

1. REFERENCES

Adegoke, A.A., Stenström, T.A., Okoh, A.I., 2017. *Stenotrophomonas maltophilia* as an emerging ubiquitous pathogen: Looking beyond contemporary antibiotic therapy. Front. Microbiol. 8, 2276. doi:10.3389/fmicb.2017.02276

Ahmad, N., Ali, S.M., Khan, A.U., 2019. Molecular characterization of novel sequence type of carbapenem-resistant New Delhi metallo-β-lactamase-1-producing *Klebsiella pneumoniae* in the neonatal intensive care unit of an Indian hospital. Int. J. Antimicrob. Agents 53, 525–529. doi:10.1016/j.ijantimicag.2018.12.005

Almuzara, M., Montaña, S., Lazzaro, T., Uong, S., Parmeciano Di Noto, G., Traglia, G., Bakai, R., Centrón, D., Iriarte, A., Quiroga, C., Ramirez, M.S., 2017. Genetic analysis of a PER-2-producing *Shewanella* sp. strain harbouring a variety of mobile genetic elements and antibiotic resistance determinants. J. Glob. Antimicrob. Resist. 11, 81–86. doi:10.1016/j.jgar.2017.06.005

Aubert, D., Naas, T., Héritier, C., Poirel, L., Nordmann, P., 2006. Functional characterization of IS*1999*, an IS*4* family element involved in mobilization and expression of β-lactam resistance genes. J. Bacteriol. 188, 6506–6514. doi:10.1128/JB.00375-06

Balbin, M.M., Hull, D., Guest, C., Nichols, L., Dunn, R., Thakur, S., 2020. Antimicrobial resistance and virulence factors profile of *Salmonella* spp. and *Escherichia coli* isolated from different environments exposed to anthropogenic activity. J. Glob. Antimicrob. Resist. 22, 578–583. doi:10.1016/j.jgar.2020.05.016

Bradley, P., den Bakker, H.C., Rocha, E.P.C., McVean, G., Iqbal, Z., 2019. Ultrafast search of all deposited bacterial and viral genomic data. Nat. Biotechnol. 37, 152–159. doi:10.1038/s41587-018-0010-1

Carrër, A., Poirel, L., Yilmaz, M., Akan, Ö.A., Feriha, C., Cuzon, G., Matar, G., Honderlick, P., Nordmann, P., 2010. Spread of OXA-48-encoding plasmid in Turkey and beyond. Antimicrob. Agents Chemother. 54, 1369–1373. doi:10.1128/AAC.01312-09

Centers for Disease Control and Prevention, 2013. Antibiotic resistance threats in the United States, 2013. doi:CS239559-B

Chah, K.F., Agbo, I.C., Eze, D.C., Somalo, S., Estepa, V., Torres, C., 2010. Antimicrobial resistance, integrons and plasmid replicon typing in multiresistant clinical *Escherichia coli* strains from Enugu State, Nigeria. J. Basic Microbiol. 50, 18–24. doi:10.1002/jobm.200900325

Dickinson, A.W., Power, A., Hansen, M.G., Brandt, K.K., Piliposian, G., Appleby, P., O'Neill, P.A., Jones, R.T., Sierocinski, P., Koskella, B., Vos, M., 2019. Heavy metal pollution and co-selection for antibiotic resistance: A microbial palaeontology approach. Environ. Int. 132, 105117. doi:10.1016/j.envint.2019.105117

ECDC, 2019. Carbapenem-resistant *Enterobacteriaceae*-second update, Ecdc.

Hansen, B., Thorling, L., Dalgaard, T., Erlandsen, M., 2011. Trend reversal of nitrate in Danish groundwater - A reflection of agricultural practices and nitrogen surpluses since 1950. Environ. Sci. Technol. 45, 228–234. doi:10.1021/es102334u

Hassan, J., Eddine, R.Z., Mann, D., Li, S., Deng, X., Saoud, I.P., Kassem, I.I., 2020. The mobile colistin resistance gene, *mcr-1.1*, is carried on IncX4 plasmids in multidrug resistant *E. coli* isolated from rainbow trout aquaculture. Microorganisms 8, 1–13. doi:10.3390/microorganisms8111636

Henriques, I., Tacão, M., Leite, L., Fidalgo, C., Araújo, S., Oliveira, C., Alves, A., 2016. Co-selection of antibiotic and metal(loid) resistance in Gram-negative epiphytic bacteria from contaminated salt marshes. Mar. Pollut. Bull. 109, 427–434. doi:10.1016/j.marpolbul.2016.05.031

Hrenovic, J., Ivankovic, T., Ivekovic, D., Repec, S., Stipanicev, D., Ganjto, M., 2017. The fate of carbapenem-resistant bacteria in a wastewater treatment plant. Water Res. 126, 232–239. doi:10.1016/j.watres.2017.09.007

Jahanbakhsh, S., Smith, M.G., Kohan-Ghadr, H.-R., Letellier, A., Abraham, S., Trott, D.J., Fairbrother, J.M., 2016. Dynamics of extended-spectrum cephalosporin resistance in pathogenic *Escherichia coli* isolated from diseased pigs in Quebec, Canada. Int. J. Antimicrob. Agents 48, 194–202. doi:10.1016/j.ijantimicag.2016.05.001

Janda, J.M., 2014. Shewanella: A marine pathogen as an emerging cause of human disease. Clin. Microbiol. Newsl. 36, 25–29. doi:10.1016/j.clinmicnews.2014.01.006

Kämpfer, P., Glaeser, S.P., Packroff, G., Behringer, K., Exner, M., Chakraborty, T., Schmithausen, R.M., Doijad, S., 2018. *Lelliottia aquatilis* sp. Nov., isolated from drinking water. Int. J. Syst. Evol. Microbiol. 68, 2454–2461. doi:10.1099/ijsem.0.002854

Kim, Y.J., Park, J.H., Seo, K.H., 2018. Presence of *Stenotrophomonas maltophilia* exhibiting high genetic similarity to clinical isolates in final effluents of pig farm wastewater treatment plants. Int. J. Hyg. Environ. Health 221, 300–307. doi:10.1016/j.ijheh.2017.12.002

Lamba, M., Ahammad, S.Z., 2017. Sewage treatment effluents in Delhi: A key contributor of β-lactam resistant bacteria and genes to the environment. Chemosphere 188, 249–256. doi:10.1016/j.chemosphere.2017.08.133

Leverstein-van Hall, M.A., Dierikx, C.M., Cohen Stuart, J., Voets, G.M., van den Munckhof, M.P., van Essen-Zandbergen, A., Platteel, T., Fluit, A.C., van de Sande-Bruinsma, N., Scharinga, J., Bonten, M.J.M., Mevius, D.J., 2011. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin. Microbiol. Infect. 17, 873–880. doi:10.1111/j.1469-0691.2011.03497.x

Liu, Z., Wang, Y., Walsh, T.R., Liu, D., Shen, Z., Zhang, R., Yin, W., Yao, H., Li, J., Shen, J., 2017. Plasmid-mediated novel *bla*$_{NDM-17}$ gene encoding a carbapenemase with enhanced activity in a sequence type 48 *Escherichia coli* strain. Antimicrob. Agents Chemother. 61. doi:10.1128/AAC.02233-16

Liu, Z., Xiao, X., Li, Y., Liu, Y., Li, R., Wang, Z., 2019. Emergence of IncX3 plasmid-harboring *bla*$_{NDM–5}$ dominated by *Escherichia coli* ST48 in a goose farm in Jiangsu, China. Front. Microbiol. 10. doi:10.3389/fmicb.2019.02002

Luo, J., Yao, X., Lv, L., Doi, Y., Huang, X., Huang, S., Liu, J.H., 2017. Emergence of *mcr-1* in *Raoultella ornithinolytica* and *Escherichia coli* isolates from retail vegetables in China. Antimicrob. Agents Chemother. 61. doi:10.1128/AAC.01139-17

Madec, J.Y., Haenni, M., Ponsin, C., Kieffer, N., Rion, E., Gassilloud, B., 2016. Sequence type 48 *Escherichia coli* carrying the *bla*$_{CTX-M-1}$ IncI1/ST3 plasmid in drinking water in France. Antimicrob. Agents Chemother. 60, 6430–6432. doi:10.1128/AAC.01135-16

Malki, M., Bouchaou, L., Hirich, A., Ait Brahim, Y., Choukr-Allah, R., 2017. Impact of agricultural practices on groundwater quality in intensive irrigated area of Chtouka-Massa, Morocco. Sci. Total Environ. 574, 760–770. doi:10.1016/j.scitotenv.2016.09.145

Manageiro, V., Ferreira, E., Caniça, M., Manaia, C.M., 2014. GES-5 among the β-lactamases detected in ubiquitous bacteria isolated from aquatic environment samples. FEMS Microbiol. Lett. 351, 64–69. doi:10.1111/1574-6968.12340

Moura, A., Soares, M., Pereira, C., Leitão, N., Henriques, I., Correia, A., 2009. INTEGRALL: A database and search engine for integrons, integrases and gene cassettes. Bioinformatics 25, 1096–1098. doi:10.1093/bioinformatics/btp105

Poirel, L., Liard, A., Nordmann, P., Mammeri, H., 2005. Origin of plasmid-

mediated quinolone resistance determinant QnrA. Antimicrob. Agents Chemother. 49, 3523–3525. doi:10.1128/AAC.49.8.3523

Poirel, L., Potron, A., Nordmann, P., 2012. OXA-48-like carbapenemases: the phantom menace. J. Antimicrob. Chemother. 67, 1597–1606. doi:10.1093/jac/dks121

Poovorawan, K., Chatsuwan, T., Lakananurak, N., Chansaenroj, J., Komolmit, P., Poovorawan, Y., 2013. *Shewanella haliotis* associated with severe soft tissue infection, Thailand, 2012. Emerg. Infect. Dis. doi:10.3201/eid1906.121607

Potron, A., Poirel, L., Nordmann, P., 2011. Origin of OXA-181, an emerging carbapenem-hydrolyzing oxacillinase, as a chromosomal gene in *Shewanella xiamenensis*. Antimicrob. Agents Chemother. 55, 4405–4407. doi:10.1128/AAC.00681-11

Ribeiro, T.G., Gonçalves, B.R., da Silva, M.S., Novais, Â., Machado, E., Carriço, J.A., Peixe, L., 2017. *Citrobacter portucalensis* sp. Nov., isolated from an aquatic sample. Int. J. Syst. Evol. Microbiol. 67, 3513–3517. doi:10.1099/ijsem.0.002154

Sato, T., Suzuki, Y., Shiraishi, T., Honda, H., Shinagawa, M., Yamamoto, S., Ogasawara, N., Takahashi, H., Takahashi, S., Tamura, Y., Yokota, S.I., 2017. Tigecycline nonsusceptibility occurs exclusively in fluoroquinolone-resistant *Escherichia coli* clinical isolates, including the major multidrug-resistant lineages O25b:H4-ST131-H30R and O1-ST648. Antimicrob. Agents Chemother. 61. doi:10.1128/AAC.01654-16

Shahada, F., Chuma, T., Kosugi, G., Kusumoto, M., Iwata, T., Akiba, M., 2013. Distribution of extended-spectrum cephalosporin resistance determinants in *Salmonella enterica* and *Escherichia coli* isolated from broilers in southern Japan. Poult. Sci. 92, 1641–9. doi:10.3382/ps.2012-02934

Smet, A., Martel, A., Persoons, D., Dewulf, J., Heyndrickx, M., Claeys, G., Lontie, M., Van Meensel, B., Herman, L., Haesebrouck, F., Butaye, P., 2010. Characterization of extended-spectrum β-lactamases produced by *Escherichia coli* isolated from hospitalized and nonhospitalized patients: Emergence of CTX-M-15-producing strains causing urinary tract infections. Microb. Drug Resist. 16, 129–134. doi:10.1089/mdr.2009.0132

Sun, Q., Wang, H., Shu, L., Dong, N., Yang, F., Zhou, H., Chen, S., Zhang, R., 2019. *Leclercia adecarboxylata* from human gut flora carries *mcr-4.3* and bla_{IMP-4}-bearing plasmids. Front. Microbiol. 10, 2805. doi:10.3389/fmicb.2019.02805

Teixeira, P., Tacão, M., Pureza, L., Gonçalves, J., Silva, A., Cruz-Schneider, M.P.,

Henriques, I., 2020. Occurrence of carbapenemase-producing *Enterobacteriaceae* in a Portuguese river: *bla*$_{NDM}$, *bla*$_{KPC}$ and *bla*$_{GES}$ among the detected genes. Environ. Pollut. 260. doi:10.1016/j.envpol.2020.113913

Tsai, M.S., You, H.L., Tang, Y.F., Liu, J.W., 2008. *Shewanella* soft tissue infection: case report and literature review. Int. J. Infect. Dis. 12, e119–e124. doi:10.1016/j.ijid.2008.03.020

Virgílio Cruz, J., Silva, M.O., Dias, M.I., Isabel Prudêncio, M., 2013. Groundwater composition and pollution due to agricultural practices at Sete Cidades volcano (Azores, Portugal). Appl. Geochemistry 29, 162–173. doi:10.1016/j.apgeochem.2012.11.009

WHO, 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. doi:10.1016/S1473-3099(09)70222-1

Xu, hui, Huo, C., Sun, Y., Zhou, Y., Xiong, Y., Zhao, Z., Zhou, Q., Sha, L., Zhang, B., Chen, Y., 2019. Emergence and molecular characterization of multidrug-resistant *Klebsiella pneumoniae* isolates harboring *bla*$_{CTX-M-15}$ extended-spectrum β-lactamases causing ventilator-associated pneumonia in China. Infect. Drug Resist. 12, 33–43. doi:10.2147/IDR.S189494

Yang, F., Mao, D., Zhou, H., Luo, Y., 2016. Prevalence and fate of carbapenemase genes in a wastewater treatment plant in northern China. PLoS One 11, e0156383. doi:10.1371/journal.pone.0156383

Zong, Z., 2011. Nosocomial peripancreatic infection associated with *Shewanella xiamenensis*. J. Med. Microbiol. 60, 1387–1390. doi:10.1099/jmm.0.031625-0

Zou, H., Berglund, B., Xu, H., Chi, X., Zhao, Q., Zhou, Z., Xia, H., Li, X., Zheng, B., 2020. Genetic characterization and virulence of a carbapenem-resistant *Raoultella ornithinolytica* isolated from well water carrying a novel megaplasmid containing *bla*$_{NDM-1}$. Environ. Pollut. 260, 114041. doi:10.1016/j.envpol.2020.114041

IV. SUPPLEMENTAL MATERIAL

CHAPTER 1: *Shewanella as origin of bla$_{OXA-48}$ genes*

TABLE S1. PCR primers used in this study.

Target	Primer Sequence (5'-3')	Reference
BOX element	BOXA1R: CTACGGCAAGGCGACGCTGACG	(Versalovic et al., 1994)
16S rDNA	27F: AGAGTTTGATCCTGGCTCAG 1492R: GGTTACCTTGTTACGACTTT	(Lane, 1991)
gyrB	SW_GyrB_F: GAAGTGGCKATGCAGTGGAA SW_GyrB_R: CGRCRAATACCACAGCCRAG	(Antonelli et al., 2015)
intI1	Int1_F: ACATGCGTGTAAATCGTC Int1_R: CTGGATTTCGATGACGGCACG	(Kraft et al., 1986)
intI2	Int2_F: ACGGCTACCCTCTGTTAT Int2_R: TTATTGCTGGGATTAGGC	(Goldstein et al., 2001)
bla$_{OXA-48}$-like	OXA48_F: GCGTGGTTAAGGATGAACAC OXA48_R: CATCAAGTTCAACCCAACCG	(Poirel et al., 2011)
bla$_{OXA-48}$-like	OXA48 54I_F: AGCAAGGATTTACCAATAAT OXA48 54I_R: GGCATATCCATATTCATC	(Zong, 2011)
bla$_{OXA-48}$-like	Set1_F: TTAGCCTTATCGGCTGT Set1_R: GGAATWATYTTTTCCTGTTT	This study
bla$_{OXA-48}$-like	Set2_F: GCCATATCGACTGTGTTG Set2_R: ACGTGTTCCAGTTTTAA	This study
lysR	LysR: AAGGGATTCTCCCAAGCTGC	(Tacão et al., 2013)
peptidase C15-encoding gene	C15_fwd: TTACGGCCTGGGAAGTGTTC	(Tacão et al., 2013)
B/O replicon	B/O-F: GCGGTCCGGAAAGCCAGAAAAC B/O-R: TCTGCGTTCCGCCAAGTTCGA	(Carattoli et al., 2005)
FIC replicon	FIC-F: GTGAACTGGCAGATGAGGAAGG FIC-R: TTCTCCTCGTCGCCAAACTAGAT	(Carattoli et al., 2005)
A/C replicon	A/C-F: GAGAACCAAAGACAAAGACCTGGA A/C-R: ACGACAAACCTGAATTGCCTCCTT	(Carattoli et al., 2005)
P replicon	P-F: CTATGGCCCTGCAAACGCGCCAGAAA P-R: TCACGCGCCAGGGCGCAGCC	(Carattoli et al., 2005)
T replicon	T-F: TTGGCCTGTTTGTGCCTAAACCAT T-R: CGTTGATTACACTTAGCTTTGGAC	(Carattoli et al., 2005)
K/B replicon	K/B-F: GCGGTCCGGAAAGCCAGAAAAC K/B-R: TCTTTCACGAGCCCGCCAAA	(Carattoli et al., 2005)
W replicon	W-F: CCTAAGAACAACAAAGCCCCCG W-R: GGTGCGCGGCATAGAACCGT	(Carattoli et al., 2005)
FIIA replicon	FIIA-F: CTGTCGTAAGCTGATGGC FIIA-R: CTCTGCCACAAACTTCAGC	(Carattoli et al., 2005)
FIA replicon	FIA-F: CCATGCTGGTTCTAGAGAAGGTG FIA-R: GTATATCCTTACTGGCTTCCGCAG	(Carattoli et al., 2005)
FIB replicon	FIB-F: GGAGTTCTGACACACGATTTTCTG FIB-R: CTCCCGTCGCTTCAGGGCATT	(Carattoli et al., 2005)
Y replicon	Y-F: AATTCAAACAACACTGTGCAGCCTG Y-R: GCGAGAATGGACGATTACAAAACTTT	(Carattoli et al., 2005)
I1 replicon	I1F: CGAAAGCCGGACGGCAGAA I1R: TCGTCGTTCCGCCAAGTTCGT	(Carattoli et al., 2005)
X replicon	X-F: AACCTTAGAGGCTATTTAAGTTGCTGAT X-R: TGAGAGTCAATTTTTATCTCATGTTTTAGC	(Carattoli et al., 2005)
HI1 replicon	HI1-F: GGAGCGATGGATTACTTCAGTAC HI1-R: TGCCGTTTCACCTCGTGAGTA	(Carattoli et al., 2005)
N replicon	N-F: GTCTAACGAGCTTACCGAAG N-R: GTTTCAACTCTGCCAAGTTC	(Carattoli et al., 2005)
HI2 replicon	HI2-F: TTTCTCCTGAGTCACCTGTTAACAC HI2-R: GGCTCACTACCGTTGTCATCCT	(Carattoli et al., 2005)
L/M replicon	L/M-F: GGATGAAAACTATCAGCATCTGAAG L/M-R: CTGCAGGGGCGATTCTTTAGG	(Carattoli et al., 2005)
Frep replicon	Frep-F: TGATCGTTTAAGGAATTTTG	(Carattoli et al., 2005)

	Frep-R: GAAGATCAGTCACACCATCC	
ColE-type	CC7059F: TTCGTGCACACAGCCCA CC7063F: GCGGACAGGTATCCGGTAA CC7062R: TGCGGTTATCCACAGAATCA	(Chen et al., 2010)
IS*1999*	IS1999A: CAGCAATTCTTTCTCCGTG IS1999B: CAAGCACAACATCAAGCGC	(Poirel et al., 2004)
IS*Ecp1*	fwd5': TTCAAAAAGCATAATCAAAGC rev: CAACCACCTTTCAATCATTTT	(Eckert et al., 2006)

TABLE S2. *In silico* analysis of draft and complete *Shewanella* genomes currently available in public databases (filled circle – present, open circle – absent; nd – not determined).

Accession number	**Affiliation**	**Strain**	**Genome status**	**C15-like gene**	**Putative bla_{OXA}**	***lysR*-like**
CDQH01000000	*S. algae*	MARS 14	Draft	●	●	●
BALO00000000	*S. algae*	JCM 21037	Draft	●	●	●
JPMA01000000	*S. algae*	C6G3	Draft	●	●	●
MDKA00000000	*S. algae*	BrY	Draft	●	●	●
MBFW00000000	*S. algae*	CSB04KR	Draft	●	●	●
CP000507	*S. amazonensis*	SB2B	Complete	●	○	●
CP002767	*S. baltica*	BA175	Draft	●	●	●
CP002811	*S. baltica*	OS117	Complete	●	●	●
CP000563	*S. baltica*	OS155	Complete	●	●	●
CP000753	*S. baltica*	OS185	Complete	●	●	●
CP000891	*S. baltica*	OS195	Complete	●	●	●
CP001252	*S. baltica*	OS223	Complete	●	●	●
CP002383	*S. baltica*	OS678	Complete	●	●	●
AECY00000000	*S. baltica*	OS183	Draft	●	●	●
AGEX00000000	*S. baltica*	OS625	Draft	●	●	●
LWED00000000	*S. baltica*	M1	Draft	●	●	●
ABIC00000000	*S. benthica*	KT99	Draft	●	○	●
JAEC00000000	*S. colwelliana*	ATCC 39565	Draft	●	●	●
MCBT00000000	*S. colwelliana*	CSB03KR	Draft	●	●	●
AXZL00000000	*S. decolorationis*	S12	Draft	●	●	●
CP000302	*S. denitrificans*	OS217	Complete	●	○	●
JADX00000000	*S. fidelis*	ATCC BAA-318	Draft	●	●	●
LRDC01000000	*S. frigidimarina*	Ag06-30	Draft	nd	nd	nd
CP000447	*S. frigidimarina*	NCIMB 400	Complete	●	○	●
CP000931	*S. halifaxensis*	HAW-EB4	Complete	●	●	●
BALL00000000	*S. haliotis*	JCM 14758	Draft	●	●	●
CP000606	*S. loihica*	PV-4	Complete	●	●	●
BALM00000000	*S. marina*	JCM 15074	Draft	●	○	●
AE014299	*S. oneidensis*	MR-1	Complete	●	●	●
CP000851	*S. pealeana*	ATCC 700345	Complete	●	●	●
CP000472	*S. piezotolerans*	WP3	Complete	●	●	●
JAEU00000000	*S. putrefaciens*	HRCR-6	Draft	●	●	●
CP002457	*S. putrefaciens**	200	Complete	●	●	●
CP000681	*S. putrefaciens*	CN-32	Complete	●	●	●

BALN00000000	*S. putrefaciens*	JCM 20190	Draft	●	●	●
JVAQ01000000	*S. sediminis*	599_SSED	Draft	●	○	●
CP000821	*S. sediminis*	HAW-EB3	Complete	nd	nd	nd
AP011177	*S. violacea*	DSS12	Complete	●	○	●
JAEH00000000	*S. waksmanii*	ATCC BAA-643	Draft	●	●	●
CP000961	*S. woodyi*	ATCC 51908	Complete	●	○	●
JGVI00000000	*S. xiamenensis*	BC01	Draft	●	●	●
LDOA00000000	*S. xiamenensis**	T17	Draft	●	●	●
AFOZ00000000	*Shewanella* sp.	HN-41	Draft	nd	nd	nd
JPEO00000000	*Shewanella* sp.	YQH10	Draft	●	○	●
CP000469	*Shewanella sp.*	ANA-3	Complete	●	●	●
CP000446	*Shewanella* sp.	MR-4	Complete	●	●	●
CP000444	*Shewanella* sp.	MR-7	Complete	●	●	●
CP000503	*Shewanella* sp.*	W3-18-1	Complete	●	●	●
JADP00000000	*Shewanella* sp.	38A_GOM-205m	Draft	●	●	●
JSFF00000000	*Shewanella* sp.	ECSMB14101	Draft	●	○	●
JWGX00000000	*Shewanella* sp.	ECSMB14102	Draft	●	●	●
BAXN01000000	*Shewanella* sp.	JCM 19057	Draft	●	●	●
LKTL01000000	*Shewanella* sp.	P1-14-1	Draft	●	○	●
AKZL00000000	*Shewanella* sp.	POL2	Draft	●	●	●
LGYY01000000	*Shewanella* sp.	Sh95	Draft	●	●	●
JTLE00000000	*Shewanella* sp.	ZOR0012	Draft	●	●	●
JPII00000000	*Shewanella* sp.	cp20	Draft	●	●	●
MPDG00000000	*Shewanella* sp.	SACH	Draft	●	●	●
LZFV00000000	*Shewanella* sp.	UCD-FRSSP16_17	Draft	●	○	●
FKJK00000000	*Shewanella* sp.	Alg231_23	Draft	●	○	●

**Shewanella* genomes with IS*1999* identified.

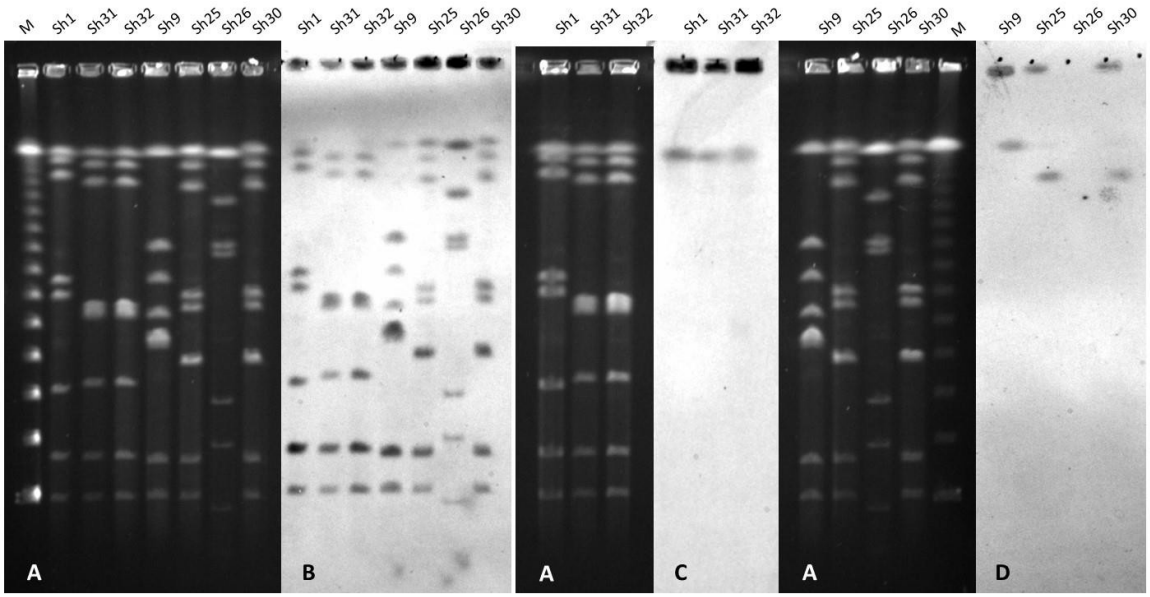


FIGURE S1. PFGE (A) and DNA hybridization results with 16S rDNA (B), or var1 (C) or var2 (D) gene probe of *Shewanella* spp. genomic DNA digested with I-*Ceu*I (BioLabs, UK); Lane M: CHEF DNA Size standard, PFGE lambda ladder (Bio-Rad, USA).

REFERENCES

Antonelli, A., Di Palo, D.M., Galano, A., Becciani, S., Montagnani, C., Pecile, P., Galli, L., Rossolini, G.M., 2015. Intestinal carriage of *Shewanella xiamenensis* simulating carriage of OXA-48–producing *Enterobacteriaceae*. Diagn. Microbiol. Infect. Dis. 82, 1–3. doi:10.1016/j.diagmicrobio.2015.02.008

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K.L., Threlfall, E.J., 2005. Identification of plasmids by PCR-based replicon typing. J. Microbiol. Methods 63, 219–228. doi:10.1016/j.mimet.2005.03.018

Chen, C.-Y., Lindsey, R.L., Strobaugh, T.P., Frye, J.G., Meinersmann, R.J., 2010. Prevalence of ColE1-like plasmids and kanamycin resistance genes in *Salmonella enterica* serovars. Appl. Environ. Microbiol. 76, 6707–14. doi:10.1128/AEM.00692-10

Eckert, C., Gautier, V., Arlet, G., 2006. DNA sequence analysis of the genetic environment of various bla_{CTX-M} genes. J. Antimicrob. Chemother. 57, 14–23. doi:10.1093/jac/dki398

Goldstein, C., Lee, M.D., Sanchez, S., Hudson, C., Phillips, B., Register, B., Grady, M., Liebert, C., Summers, A.O., White, D.G., Maurer, J.J., 2001. Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. Antimicrob. Agents Chemother. 45, 723–726. doi:10.1128/AAC.45.3.723

Kraft, C.A., Timbury, M.C., Platt, D.J., 1986. Distribution and genetic location of Tn7 in trimethoprim-resistant *Escherichia coli*. J. Med. Microbiol. 22, 125–131.

Lane, D., 1991. 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics, in: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York, pp. 115–175.

Poirel, L., Héritier, C., Tolün, V., Nordmann, P., 2004. Emergence of oxacillinase-mediated resistance to imipenem in *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. 48, 15–22. doi:10.1128/AAC.48.1.15-22.2004

Poirel, L., Walsh, T.R., Cuvillier, V., Nordmann, P., 2011. Multiplex PCR for detection of acquired carbapenemase genes. Diagn. Microbiol. Infect. Dis. 70, 119–223. doi:10.1016/j.diagmicrobio.2010.12.002

Tacão, M., Correia, A., Henriques, I., 2013. Environmental *Shewanella xiamenensis* strains that carry *bla*$_{OXA-48}$ or *bla*$_{OXA-204}$ genes: additional proof for *bla*$_{OXA-48}$-like gene origin. Antimicrob. Agents Chemother. 57, 6399–6400. doi:10.1128/AAC.00771-13

Versalovic, J., Schneider, M., De Bruijn, F.J., Lupski, J.R., 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol. Cell. Biol. 5, 25–40.

Zong, Z., 2011. Nosocomial peripancreatic infection associated with *Shewanella xiamenensis*. J. Med. Microbiol. 60, 1387–1390. doi:10.1099/jmm.0.031625-0

CHAPTER 2: *Shewanella as origin of qnrA genes*

TABLE S1. Environmental strains used in this study, isolation source and affiliation (Azevedo et al., 2012; Fidalgo et al., 2016; Tacão et al., 2018). The QnrA variants detected in this study are included.

Strain	Source[a]	Affiliation	QnrA[b]
Sh1	SP	*S. xiamenensis*	ND
Sh2	SP	*S. algae*	QnrA12
Sh3	SP	*Shewanella fodinae*	ND
Sh4	SP	*S. haliotis*	nv11
Sh5	SP	*S. xiamenensis*	ND
Sh6	SP	*Shewanella* sp.	ND
Sh7	SP	*Shewanella* sp.	ND
Sh8	CL	*Shewanella aestuarii*	ND
Sh9	CL	*Shewanella baltica*	ND
Sh10	CL	*Shewanella hafniensis*	ND
Sh11	CL	*S. aestuarii*	ND
Sh12	CL	*S. aestuarii*	ND
Sh13	CL	*S. aestuarii*	ND
Sh14	CL	*S. haliotis*	QnrA3
Sh15	CL	*S. indica*	QnrA2
Sh16	CL	*S. indica*	QnrA2
Sh17	EW	*S. hafniensis*	ND
Sh18	EW	*S. hafniensis*	ND
Sh19	EW	*S. baltica*	ND
Sh20	EW	*S. hafniensis*	ND
Sh21	EW	*S. baltica*	ND
Sh22	EW	*S. baltica*	ND
Sh23	EW	*S. putrefaciens*	ND
Sh24	EW	*S. hafniensis*	ND
Sh25	EW	*S. hafniensis*	ND
Sh26	EW	*S. hafniensis*	ND
Sh27	EW	*S. putrefaciens*	ND
Sh28	EW	*Shewanella algidipiscicola*	ND
Sh29	EW	*S. hafniensis*	ND
Sh30	EW	*S. hafniensis*	ND
Sh31	RW	*S. xiamenensis*	ND
Sh32	RW	*S. xiamenensis*	ND
Sh33	RW	*S. xiamenensis*	ND

[a]SP - saltmarsh plant, CL - cockle, EW - estuarine water, RW - river water.

[b]ND, not detected.

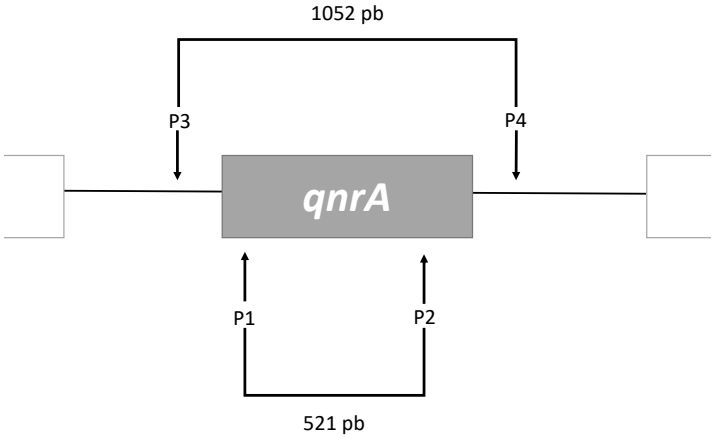


FIGURE S1. Schematic representation of target regions and expected amplicon size for each primer combination; P1/P2 (Guillard et al., 2011) and P3/P4 (this study).

REFERENCES:

Azevedo, J.S.N., Ramos, I., Araújo, S., Oliveira, C.S., Correia, A., Henriques, I.S., 2012. Spatial and temporal analysis of estuarine bacterioneuston and bacterioplankton using culture-dependent and culture-independent methodologies. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 101, 819–835. doi:10.1007/s10482-012-9697-z

Fidalgo, C., Henriques, I., Rocha, J., Tacão, M., Alves, A., 2016. Culturable endophytic bacteria from the salt marsh plant *Halimione portulacoides*: phylogenetic diversity, functional characterization, and influence of metal(loid) contamination. Environ. Sci. Pollut. Res. 23, 10200–10214. doi:10.1007/s11356-016-6208-1

Guillard, T., Moret, H., Brasme, L., Carlier, A., Vernet-Garnier, V., Cambau, E., de Champs, C., 2011. Rapid detection of *qnr* and *qepA* plasmid-mediated quinolone resistance genes using real-time PCR. Diagn. Microbiol. Infect. Dis. 70, 253–259. doi:10.1016/j.diagmicrobio.2011.01.004

Tacão, M., Araújo, S., Vendas, M., Alves, A., Henriques, I., 2018. *Shewanella* species as the origin of $bla_{\text{OXA-48}}$ genes: insights into gene diversity, associated phenotypes and possible transfer mechanisms. Int. J. Antimicrob. Agents 51, 340–348. doi:10.1016/j.ijantimicag.2017.05.014

CHAPTER 3: *Escherichia coli in water and vegetables*

FIGURE S1. Map of the sampling area with the location of the chemical complex of Estarreja and the 16 sampling sites (WGS84 coordinates system).

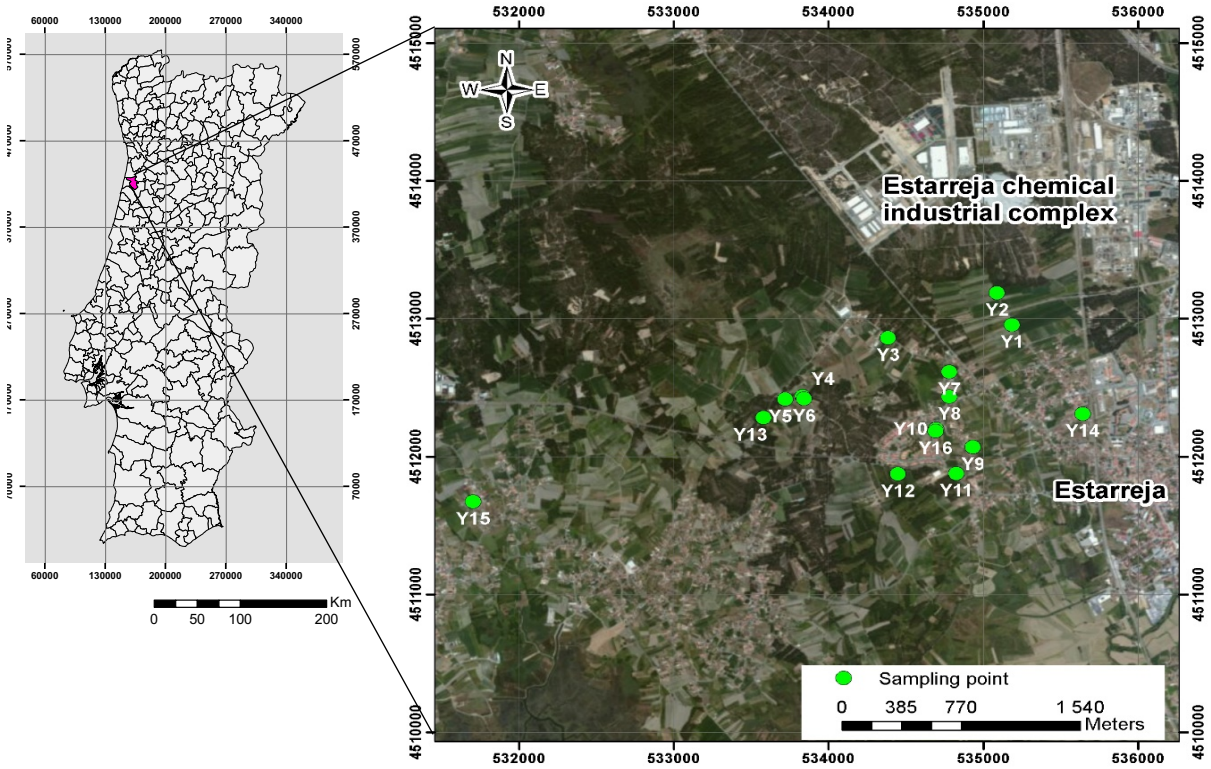


TABLE S1. Number of isolates obtained per site and number of distinct BOX-PCR profiles.

Site	No. of selected *E. coli* isolates (n=449) from water (W) and vegetables (V)	No. of *E. coli* representative isolates with different BOX-PCR profiles (n=139)
Y1	0	0
Y2	0	0
Y3	0	0
Y4	30W	10W
Y5	15W	6W
Y6	0	0
Y7	5V	4V
Y8	0	0
Y9	13W, 11V	10W, 1V
Y10	88W, 71V	30W, 3V
Y11	0	0
Y12	1W, 33V	1W, 6V
Y13	1W	1W
Y14	0	0
Y15	7W, 105V	6W, 36V
Y16	55W, 14V	19W, 6V

TABLE S2. Primers and PCR conditions used in this study.

Gene targeted	Primers name and sequence[1] (5'-3')	Amplicon size (bp)	Reference
bla$_{TEM}$	TEM_F: AAAGATGCTGAAGATCA TEM_R: TTTGGTATGGCTTCATTC	425	Speldooren et al., 1998
bla$_{SHV}$	SHV_F: GCGAAAGCCAGCTGTCGGGC SHV_R: GATTGGCGGCGCTGTTATCGC	304	Henriques et al., 2006
bla$_{CTX-M}$	CTX_F: SCVATGTGCAGYACCAGTAA CTX_R: GCTGCCGGTYTTATCVCC	652	Lu et al., 2010
bla$_{IMP}$	IMP_F: GAATAGAGTGGCTTAATTGTC IMP_R: GGTTTAAYAAAACAACCACC	232	Henriques et al., 2006
bla$_{VIM}$	VIM_F: GATGGTGTTTGGTCGCATATCG VIM_R: GCCACGTTCCCCGCAGACG	475	Henriques et al., 2006
bla$_{OXA-48}$-like	OXA48_ALL_F: GCGTGTATTAGCCTTATCGGC OXA48_ALL_R: CTAGGGAATAATTTTTTCCTGTTTG	750	This study
bla$_{KPC}$	KPC_F: CATTCAAGGGCTTTCTTGCTGC KPC_R: ACGACGGCATAGTCATTTGC	538	Dallenne et al., 2010
bla$_{GES}$	GES_F: AGTCGGCTAGACCGGAAAG GES_R: TTTGTCCGTGCTCAGGAT	399	Dallenne et al., 2010
bla$_{AmpC}$-like	ACC_F: CACCTCCAGCGACTTGTTAC ACC_R: GTTAGCCAGCATCACGATCC FOX_F: CTACAGTGCGGGTGGTTT FOX_R: CTATTTGCGGCCAGGTGA MOX_F: GCAACAACGACAATCCATCCT MOX_R: GGGATAGGCGTAACTCTCCCAA DHA_F: TGATGGCACAGCAGGATATTC DHA_R: GCTTTGACTCTTTCGGTATTCG CIT_F: CGAAGAGGCAATGACCAGAC CIT_R: ACGGACAGGGTTAGGATAGY EBC_F: CGGTAAAGCCGATGTTGCG EBC_R: AGCCTAACCCCTGATACA	346 162 895 997 538 683	Dallenne et al., 2010
tet(A)	tetA_F: GCTACATCCTGCCTTC tetA_R: GCATAGATCGGAAGAG	211	Nawaz et al., 2006
tet(B)	tetB_F: TCATTGCCGACCTCAG tetB_R: CCAACCATCACCATCC	391	Nawaz et al., 2006
tet(C)	tetC_F: CTGCTCGCTTCGCTACTTG tetC_R: GCCTACAATCCATGCCAACC	897	Nawaz et al., 2006
tet(D)	tetD_F: TGTGCTGTGGATGTTGTATCTC tetD_R: CAGTGCCGTGCCAATCAG	844	Nawaz et al., 2006
tet(E)	tetE_F: ATGAACCGCACTGTGATGATG tetE_R: ACCGACCATTACGCCATCC	744	Nawaz et al., 2006
tet(M)	tetM_F: GTGGACAAAGGTACAACGAG tetM_R: CGGTAAAGTTCGTCACACAC	406	Warsa et al., 1995
qnrA	qnrA_F: 5'-TTCTCACGCCAGGATTTG-3' qnrA_R: 5'-CCATCCAGATCGGCAAA-3'	521	Guillard et al., 2011
qnrB	qnrB_F: 5'-GGMATHGAAATTCGCCACTG-3' qnrB_R: 5'-TTYGCBGYYCGCCAGTCG-3'	261	Guillard et al., 2011
qnrS	qnrS_F: 5'-GCAAGTTCATTGAACAGGGT-3' qnrS_R: 5'-TCTAAACCGTCGAGTTCGGCG-3'	428	Cattoir et al., 2007
gyrA	gyrA-F: 5'-AAATCTGCCCGTGTCGTTGGT-3' gyrA-R: 5'-GCCATACCTACGGCGATACC-3'	314	Rodríguez-Martínez et al., 2006
parC	parC-F: 5'-CTGAATGCCAGCGCCAAATT-3' parC-R: 5'-GCGAACGATTTCGGATCGTC-3'	168	Rodríguez-Martínez et al., 2006

sul1	sul1_F: 5'-CTGAACGATATCCAAGGATTYCC-3' sul1_R: 5'-AAAAATCCCACGGRTC-3'	239	Heuer and Smalla, 2007
sul2	sul2_F: 5'-GCGCTCAAGGCAGATGGCAT-3' sul2_R: 5'-GCGTTTGATACCGGCACCCG-3'	293	Kerrn et al., 2002
sul3	sul3_F: 5'-ATTAATGATATTCAAGGTTTYCC-3' sul3_R: 5'-AAGAAGCCCATACCCGGRTC-3'	236	Heuer and Smalla, 2007
dfrA1	dfr_F: 5'-GTGAAACTATCACTAATGG-3' dfr_R: 5'-TTAACCCTTTTGCCAGATTT-3'	474	Navia et al., 2003
aadA1	aadA1_F: 5'-TATCAGAGGTAGTTGGCGTCAT-3' aadA1_R: 5'-GTTCCATAGCGTTAAGGTTTCATT-3'	486	Randall et al., 2004
aadA2	aadA2_F: 5'-TGTTGGTTACTGTGGCCGTA-3' aadA2_R: 5'-GATCTCGCCTTTCACAAAGC-3'	712	Randall et al., 2004
aadB	aadB_F: 5'-GAGCGAAATCTGCCGCTCTGG-3' aadB_R: 5'-CTGTTACAACGGACTGGCCGC-3'	371	Randall et al., 2004
strA/B	strA_F: ATGGTGGACCCTAAAACTCT strB_R: CGTCTAGGATCGAGACAAAG	893	Kozak et al., 2009
aac-cr	aac(6')-Ib-F: TTGCGATGCTCTATGAGTGGCTA aac(6')-Ib-R: CTCGAATGCCTGGCGTGTTT	482	Park et al., 2006
IntI1	IntI1_F: CCTCCCGCACGATGATC IntI1_R: TCCACGCATCGTCAGGC	280	Kraft et al., 1986
IntI1	IntI1_894F (ER.1.6F): CCCAGTGGACATAAGCCTG	variable	Moura et al., 2012
IntI2	IntI2_F: TTATTGCTGGGATTAGGC IntI2_R: ACGGCTACCCTCTGTTATC	233	Goldstein et al., 2001
attI1/3' CS	5'CS: GGCATCCAAGCAGCAAG 3'CS: AAGCAGACTTGACCTGA	integron variable region	Levesque et al., 1995
attI2/ybeA	hep74: CGGGATCCCGGACGGCATGCACGATTTGTA hep51: GATGCCATCGCAAGTACGAG	integron variable region	White et al., 2001
qacE/qacEdelta1	qacE_R: CAAGCTTTTGCCCATGAAGC	integron variable region	Sandvang et al., 1997
tniC	tniC_R (RH506): TTCAGCCGCATAAATGGAG	integron variable region	Post et al., 2007
stx1	STX1_F: ATAAATCGCCATTCGTTGACTAC STX1_R: AGAACGCCCACTGAGATCCATC	180	Paton and Paton, 1998
stx2	STX2_F: GGCACTGTCTGAAACTGCTCC STX2_R: TCGCCAGTTATCTGACATTCTG	255	Paton and Paton, 1998
eae	EAE_F: GACCCGGCACAAGCATAAGC EAE_R: CCACCTGCAGCAACAAGAGG	384	Paton and Paton, 1998
ipaH	ipaH1_F: GTTCCTTGACCGCCTTTCCGATACCGTC ipaH2_R: GCCGGTCAGCCACCCTCTGAGAGTAC	600	Aranda et al., 2007
aggR	aggRks1_F: GTATACACAAAAGAAGGAAGC aggRks2_R: ACAGAATCGTCAGCATCAGC	254	Aranda et al., 2007
elt	LT_F: GGCGACAGATTATACCGTGC LT_R: CGGTCTCTATATTCCCTGTT	450	Aranda et al., 2007

[1]F -forward; R - reverse.

[2]Primer combinations or primers used for amplification of integrons variable regions.

TABLE S3. Biochemical and bacteriological quality of irrigation water with regard to parameters included in Portuguese legislation (Ministério do Ambiente, 1998). Available online at: https://ars.els-cdn.com/content/image/1-s2.0-S016816051730288X-mmc1.pdf or https://uapt33090-my.sharepoint.com/:x:/g/personal/susana_araujo_ua_pt/EfzAmNjctmBGmnt1q4AX4ScBo9P9Fso9qoFGHO2glYR_Ug?e=Uu3c0A (until PhD presentation).

REFERENCES:

Aranda, K.R.S., Fabbricotti, S.H., Fagundes-Neto, U., Scaletsky, I.C.A., 2007. Single multiplex assay to identify simultaneously enteropathogenic, enteroaggregative, enterotoxigenic, enteroinvasive and Shiga toxin-producing *Escherichia coli* strains in Brazilian children. FEMS Microbiol. Lett. 267, 145–150. doi:10.1111/j.1574- 6968.2006.00580.x

Cattoir, V., Poirel, L., Rotimi, V., Soussy, C.J., Nordmann, P., 2007. Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates. J. Antimicrob. Chemother. 60, 394–397. doi:10.1093/jac/dkm204

Dallenne, C., Da Costa, A., Decre, D., Favier, C., Arlet, G., 2010. Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in *Enterobacteriaceae*. J. Antimicrob. Chemother. 65, 490–495. doi:10.1093/jac/dkp498

Goldstein, C., Lee, M.D., Sanchez, S., Hudson, C., Phillips, B., Register, B., Grady, M., Liebert, C., Summers, A.O., White, D.G., Maurer, J.J., 2001. Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. Antimicrob. Agents Chemother. 45, 723–726. doi:10.1128/AAC.45.3.723

Guillard, T., Moret, H., Brasme, L., Carlier, A., Vernet-Garnier, V., Cambau, E., de Champs, C., 2011. Rapid detection of *qnr* and *qepA* plasmid-mediated quinolone resistance genes using real-time PCR. Diagn. Microbiol. Infect. Dis. 70, 253–259. doi:10.1016/j.diagmicrobio.2011.01.004

Henriques, I.S., Fonseca, F., Alves, A., Saavedra, M.J., Correia, A., 2006. Occurrence and diversity of integrons and β-lactamase genes among

ampicillin-resistant isolates from estuarine waters. Res. Microbiol. 157, 938–947. doi:10.1016/j.resmic.2006.09.003

Heuer, H., Smalla, K., 2007. Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environ. Microbiol. 9, 657– 666. doi:10.1111/j.1462-2920.2006.01185.x

Kerrn, M.B., Klemmensen, T., Frimodt-Moller, N., Espersen, F., 2002. Susceptibility of Danish *Escherichia coli* strains isolated from urinary tract infections and bacteraemia, and distribution of sul genes conferring sulphonamide resistance. J. Antimicrob. Chemother. 50, 513–516. doi:10.1093/jac/dkf164

Kozak, G.K., Boerlin, P., Janecko, N., Reid-Smith, R.J., Jardine, C., 2009. Antimicrobial resistance in *Escherichia coli* isolates from swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario, Canada. Appl. Environ. Microbiol. 75, 559–566. doi:10.1128/AEM.01821-08

Kraft, C.A., Timbury, M.C., Platt, D.J., 1986. Distribution and genetic location of Tn7 in trimethoprim-resistant Escherichia coli. J. Med. Microbiol. 22, 125–131.

Levesque, C., Piche, L., Larose, C., Roy, P.H., 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents Chemother. 39, 185–191. doi:10.1128/AAC.39.1.185

Lu, S.-Y., Zhang, Y.-L., Geng, S.-N., Li, T.-Y., Ye, Z.-M., Zhang, D.-S., Zou, F., Zhou, H.-W., 2010. High diversity of extended-spectrum beta-lactamase-producing bacteria in an urban river sediment habitat. Appl. Environ. Microbiol. 76, 5972– 5976. doi:10.1128/AEM.00711-10

Ministério do Ambiente, 1998. Decreto-Lei n.º 236/1998 de 1 de Agosto, Diário da Républica.

Moura, A., Pereira, C., Henriques, I., Correia, A., 2012. Novel gene cassettes and integrons in antibiotic-resistant bacteria isolated from urban wastewaters. Res. Microbiol. 163, 92–100. doi:10.1016/j.resmic.2011.10.010

Navia, M.M., Ruiz, J., Sanchez-Cespedes, J., Vila, J., 2003. Detection of dihydrofolate reductase genes by PCR and RFLP. Diagn. Microbiol. Infect. Dis. 46, 295–298. doi:10.1016/S0732-8893(03)00062-2

Nawaz, M., Sung, K., Khan, S.A., Khan, A.A., Steele, R., 2006. Biochemical and molecular characterization of tetracycline-resistant *Aeromonas veronii* isolates from catfish. Appl. Environ. Microbiol. 72, 6461–6466. doi:10.1128/AEM.00271- 06

Park, C.H., Robicsek, A., Jacoby, G.A., Sahm, D., Hooper, D.C., 2006. Prevalence in the United States of aac(6')-Ib-cr encoding a ciprofloxacin-modifying enzyme. Antimicrob. Agents Chemother. 50, 3953–3955. doi:10.1128/AAC.00915-06

Paton, A., Paton, J., 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex assays for *stx1*, *stx2*, *eaeA*, enterohemorrhagic *E. coli hlyA*, *rfbO111*, and *rfbO157*. J. Clin. Microbiol. 36, 598–602.

Post, V., Recchia, G.D., Hall, R.M., 2007. Detection of gene cassettes in Tn402-like class 1 integrons. Antimicrob. Agents Chemother. 51, 3467–3468. doi:10.1128/AAC.00220-07

Randall, L.P., Cooles, S.W., Osborn, M.K., Piddock, L.J. V., Woodward, M.J., 2004. Antibiotic resistance genes, integrons and multiple antibiotic resistance in thirty- five serotypes of *Salmonella enterica* isolated from humans and animals in the UK. J. Antimicrob. Chemother. 53, 208–216. doi:10.1093/jac/dkh070

Rodríguez-Martínez, J.M., Velasco, C., Pascual, A., García, I., Martínez-Martínez, L., 2006. Correlation of quinolone resistance levels and differences in basal and quinolone-induced expression from three qnrA-containing plasmids. Clin. Microbiol. Infect. 12, 440–445. doi:10.1111/j.1469-0691.2006.01389.x

Sandvang, D., Aarestrup, F.M., Jensen, L.B., 1997. Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. FEMS Microbiol. Lett. 157, 177–181. doi:10.1016/S0378- 1097(97)00473-4

Speldooren, V., Heym, B., Labia, R., Nicolas-Chanoine, M.H., 1998. Discriminatory detection of inhibitor-resistant β-lactamases in *Escherichia coli* by single-strand conformation polymorphism-PCR. Antimicrob. Agents Chemother. 42, 879–884.

Warsa, U.C., Nonoyama, M., Ida, T., Okamoto, R., Okubo, T., Shimauchi, C., Kuga, A., Inoue, M., 1995. Detection of *tet*(K) and *tet*(M) in *Staphylococcus aureus* of Asian Countries by the Polymerase Chain Reaction 49, 1127–1132.

White, P.A., McIver, C.J., Rawlinson, W.D., 2001. Integrons and gene cassettes in the *Enterobacteriaceae.* Antimicrob. Agents Chemother. 45, 2658–2661. doi:10.1128/AAC.45.9.2658-2661.2001

CHAPTER 4: Multidrug-resistant *Escherichia coli* genomes

TABLE S1. List of all *E. coli* genome sequences used in this work with information on pathotype, strain designation, GenBank accession number, sero- and sequence type.

E coli	**Strain**	**GenBank accession number**	**Additional information**	
			Serotype	**ST**
EAEC	55989	NC_011748	O104:H4	678
EAEC	042	NC_017626	O44:H18	414
EHEC	12009	NC_013353	O103:H2	17
EHEC	EC4115	NC_011353	O157:H7	11
EHEC	Sakai	NC_002695	O157:H7	11
EPEC	E2348/69	NC_011601	O127:H6	15
EPEC	CB9615	NC_013941	O55:H7	335
ETEC	UMNK88	NC_017641	O149:H10	100
ETEC	E24377A	NC_009801	O_:H28	1132
ETEC	H10407	NC_017633	O78:H11	48
NMEC	CE10	NC_017646	O7:H45	62
NMEC	IHE3034	NC_017628	O18:H7:K1	95
UPEC	536	NC_008253	O6:H31	127
UPEC	CFT073	NC_004431	O6:H1	73
stxEAEC	2009EL_2071	NC_018650	O104:H4	678
stxEAEC	2009EL_2050	NC_018661	O104:H4	678
non-pathogenic	K-12 substr. MG1655	NC_000913	O16:H48	10
Unknown	Y15 V.22	WTST00000000	O8:H9	48
Unknown	Y15 V.54	WTSU00000000	O8:H9	48

TABLE S2. Virulence factors predicted against VFDB based on whole-genome analysis of *E. coli* strains isolated from lettuce (Y15 V.22 and Y15 V.54) and representatives of *E. coli* enterotoxigenic *E. coli* (ETEC), H10407, UMNK88 and E24377A.

VFclass	Virulence factors		*E. coli* Y15 V.22	*E. coli* Y15 V.54	*E.coli* H10407 (ETEC)	*E.coli* UMNK88 (ETEC)	*E.coli* E24377A (ETEC)
Adherence	AAF/II fimbriae	*aafA*	-	-	-	-	-
		aafB	-	-	-	-	-
		aafC	-	-	-	-	-
		aafD	-	-	-	-	-
	AAF/III fimbriae	*agg3A*	-	-	-	-	-
		agg3B	-	-	-	-	-
		agg3C	-	-	-	-	-
		agg3D	-	-	-	-	-
	Afimbrial adhesin AFA-I	*afaA*	-	-	-	-	-
		afaB	-	-	-	-	-
		afaC	-	-	-	-	-
		afaD	-	-	-	-	-
		afaE	-	-	-	-	-
		draP	-	-	-	-	-
	CFA/I fimbriae	*cfaA*	-	-	+	-	+
		cfaB	-	-	+	-	+
		cfaC	-	-	+	-	+
		cfaD/cfaE	-	-	+	-	+
	Curli fibers	*cgsD*	-	-	-	-	-
		cgsE	-	-	-	-	-
		cgsF	-	-	-	-	-
		cgsG	-	-	-	-	-
		csgA	-	-	-	-	-
		csgB	-	-	-	-	-
		csgC	-	-	-	-	-
	Dispersin	*aap*	-	-	-	-	-
	E. coli common pilus (ECP)	*ecpA*	-	-	+	+	+
		ecpB	-	-	+	+	+
		ecpC	-	-	+	+	+
		ecpD	-	-	+	+	+
		ecpE	-	-	+	+	+
		ecpR	-	-	+	+	+
	E.coli laminin-binding fimbriae (ELF)	*elfA*	-	+	+	+	+
		elfC	+	+	+	+	+
		elfD	-	-	+	+	+

		elfG	+	+	+	+	+
	EaeH	*eaeH*	-	-	+	+	+
	EtpA	*etpA*	-	-	+	-	-
	F1C fimbriae	*focA*	-	-	-	-	-
		focC	-	-	-	-	-
		focD	-	-	-	-	-
		focF	-	-	-	-	-
		focG	-	-	-	-	-
		focH	-	-	-	-	-
		focI	-	-	-	-	-
	Hemorrhagic *E.coli* pilus (HCP)	*hcpA*	-	+	+	+	+
		hcpB	+	+	+	+	+
		hcpC	+	+	+	+	-
	Intimin	*eae*	-	-	-	+	-
	K88 fimbriae	*faeC*	-	-	-	+	-
		faeD	-	-	-	+	-
		faeE	-	-	-	+	-
		faeF	-	-	-	+	-
		faeG	-	-	-	+	-
		faeH	-	-	-	+	-
		faeI	-	-	-	+	-
		faeJ	-	-	-	+	-
	P fimbriae	*papA*	-	-	-	-	-
		papB	-	-	-	-	-
		papC	-	-	-	-	-
		papD	-	-	-	-	-
		papE	-	-	-	-	-
		papF	-	-	-	-	-
		papG	-	-	-	-	-
		papH	-	-	-	-	-
		papI	-	-	-	-	-
		papJ	-	-	-	-	-
		papK	-	-	-	-	-
		papX	-	-	-	-	-
	Porcine attaching-effacing associated protein	*paa*	-	-	-	-	-
	S fimbriae	*sfaA*	-	-	-	-	-
		sfaB	-	-	-	-	-
		sfaC	-	-	-	-	-
		sfaD	-	-	-	-	-
		sfaE	-	-	-	-	-

		sfaF	-	-	-	-	-
		sfaG	-	-	-	-	-
		sfaH	-	-	-	-	-
		sfaS	-	-	-	-	-
	ToxB	*toxB*	-	-	-	-	-
	Type I fimbriae	*fimA*	-	+	+	-	-
		fimB	-	-	+	-	-
		fimC	-	+	+	-	-
		fimD	+	+	+	-	-
		fimE	-	-	+	-	-
		fimF	-	+	+	-	+
		fimG	-	+	+	-	+
		fimH	+	+	+	-	+
		fimI	-	+	+	-	-
Autotransporter	AIDA-I type	*tibA*	-	-	+	-	-
	AIDA-I	*aida*	-	-	-	-	-
	AatA	*aatA*	+	+	-	-	-
	Antigen 43	*agn43*	-	-	-	-	-
	Cah	*cah*	-	-	+	+	+
	Contact-dependent inhibition CDI system	*cdiA*	-	-	-	-	-
		cdiB	-	-	-	-	-
	EhaA	*ehaA*	-	-	-	-	+
	EhaB	*ehaB*	+	+	-	+	+
	Enteroaggregative immunoglobulin repeat protein	*air/eaeX*	-	-	-	-	-
	EspC	*espC*	-	-	-	-	-
	EspI	*espI*	-	-	-	-	-
	EspP	*espP*	-	-	-	-	-
	Pet	*pet*	-	-	-	-	-
	Pic	*pic*	-	-	+	-	-
	Sat	*sat*	-	-	-	-	-
	Temperature-sensitive hemagglutinin	*tsh*	-	-	-	-	-
	UpaG adhesin	*upaG/ehaG*	-	-	-	-	+
	UpaH	*upaH*	-	-	-	-	-
	Vacuolating autotransporter gene	*vat*	-	-	-	-	-
Invasion	Invasion of brain endothelial cells (Ibes)	*ibeA*	-	-	-	-	-
		ibeB	+	+	+	+	+
		ibeC	+	+	+	+	+
	Tia/Hek	*tia*	-	-	+	-	-

Iron uptake	Aerobactin siderophore	*iucA*	-	-	-	-	-
		iucB	-	-	-	-	-
		iucC	-	-	-	-	-
		iucD	-	-	-	-	-
		iutA	-	-	-	-	-
	Heme uptake	*chuA*	-	-	-	-	-
		chuS	-	-	-	-	-
		chuT	-	-	-	-	-
		chuU	-	-	-	-	-
		chuW	-	-	-	-	-
		chuX	-	-	-	-	-
		chuY	-	-	-	-	-
	Iron-regulated element	*ireA*	-	-	-	-	-
	Iron/manganese transport	*sitA*	-	-	-	-	-
		sitB	-	-	-	-	-
		sitC	-	-	-	-	-
		sitD	-	-	-	-	-
	Salmochelin siderophore	*iroB*	-	-	-	-	-
		iroC	-	-	-	-	-
		iroD	-	-	-	-	-
		iroE	-	-	-	-	-
		iroN	-	-	-	-	-
	Yersiniabactin siderophore	*fyuA*	-	-	+	-	-
		irp1	-	-	+	-	-
		irp2	-	-	+	-	-
		ybtA	-	-	+	-	-
		ybtE	-	-	+	-	-
		ybtP	-	-	+	-	-
		ybtQ	-	-	+	-	-
		ybtS	-	-	+	-	-
		ybtT	-	-	+	-	-
		ybtU	-	-	+	-	-
		ybtX	-	-	+	-	-
LEE-encoded TTSS effectors	EspB	*espB*	-	-	-	-	-
	EspF	*espF*	-	-	-	-	-
	EspG	*espG*	-	-	-	-	-
	EspH	*espH*	-	-	-	-	-
	Mitochondria-associated protein Map	*map*	-	-	-	-	-
	SepZ/EspZ	*sepZ*	-	-	-	-	-
	Tir	*tir*	-	-	-	-	-

	Cell-cycle-inhibitory factor Cif	*cif*	-	-	-	-	-
	EspFu/TccP (Tir cytoskeleton coupling protein)	*espFu/tccP*	-	-	-	-	-
	EspG2 (EPEC EspC island)	*espG2*	-	-	-	-	-
	EspJ	*espJ*	-	-	-	-	-
	EspK	*espK*	-	-	-	-	-
	EspL1	*espL1*	+	+	-	-	-
	EspL2	*espL2*	-	-	-	-	-
	EspL4	*espL4*	+	+	-	-	-
	EspM1	*espM1*	-	-	-	-	-
	EspM2	*espM2*	-	-	-	-	-
	EspN	*espN*	-	-	-	-	-
	EspO1-1	*espO101*	-	-	-	-	-
	EspO1-2	*espO102*	-	-	-	-	-
	EspR1	*espR1*	-	-	-	-	-
	EspR3	*espR3*	-	-	-	-	-
	EspR4	*espR4*	-	-	-	-	-
	EspV	*espV*	-	-	-	-	-
Non-LEE encoded TTSS effectors	EspW	*espW*	-	-	-	-	-
	EspX1	*espX1*	-	-	-	-	-
	EspX2	*espX2*	-	-	-	-	-
	EspX4	*espX4*	+	+	-	-	-
	EspX5	*espX5*	+	+	-	-	-
	EspX6	*espX6*	-	-	-	-	-
	EspX7	*espX7*	-	-	-	-	-
	EspY1	*espY1*	+	-	-	-	-
	EspY2	*espY2*	-	-	-	-	-
	EspY3	*espY3*	-	-	-	-	-
	EspY4	*espY4*	-	-	-	-	-
	EspY5	*espY5*	-	-	-	-	-
	LifA/Efa1	*lifA/efa1*	-	-	-	-	-
	NleA	*nleA*	-	-	-	-	-
	NleB1	*nleB1*	-	-	-	-	-
	NleB2-1	*nleB201*	-	-	-	-	-
	NleB2-2	*nleB202*	-	-	-	-	-
	NleC	*nleC*	-	-	-	-	-
	NleD	*nleD*	-	-	-	-	-
	NleE-1	*nleE01*	-	-	-	-	-
	NleE-2	*nleE02*	-	-	-	-	-

	NleF	*nleF*	-	-	-	-	-
	NleG-1	*nleG01*	-	-	-	-	-
	NleG-2	*nleG02*	-	-	-	-	-
	NleG-3	*nleG03*	-	-	-	-	-
	NleG2-2	*nleG202*	-	-	-	-	-
	NleG2-3	*nleG203*	-	-	-	-	-
	NleG2-4	*nleG204*	-	-	-	-	-
	NleG5-1	*nleG501*	-	-	-	-	-
	NleG5-2	*nleG502*	-	-	-	-	-
	NleG6-1	*nleG601*	-	-	-	-	-
	NleG6-2	*nleG602*	-	-	-	-	-
	NleG6-3	*nleG603*	-	-	-	-	-
	NleG7	*nleG7*	-	-	-	-	-
	NleG8-2	*nleG802*	-	-	-	-	-
	NleH1-1	*nleH101*	-	-	-	-	-
	NleH1-2	*nleH102*	-	-	-	-	-
	TccP2	*tccP2*	-	-	-	-	-
Regulation	AggR	*aggR*	-	-	-	-	-
Secretion system	AAI/SCI-II T6SS	*aaiA*	-	-	-	-	-
		aaiB	-	-	-	-	-
		aaiC/hcp	-	-	-	-	-
		aaiD	-	-	-	-	-
		aaiE	-	-	-	-	-
		aaiF	-	-	-	-	-
		aaiH	-	-	-	-	-
		aaiI	-	-	-	-	-
		aaiJ	-	-	-	-	-
		aaiK	-	-	-	-	-
		aaiL	-	-	-	-	-
		aaiM	-	-	-	-	-
		aaiN	-	-	-	-	-
		clpV/aaiP	-	-	-	-	-
		icmF/aaiO	-	-	-	-	-
		vgrG	-	-	-	-	-
	ABC transporter for dispersin	*aatA*	-	-	+	-	+
		aatB	-	-	+	-	+
		aatC	-	-	+	-	-
		aatD	-	-	+	-	+
		aatP	-	-	+	-	+
	ACE T6SS	*Undetermined*	-	-	-	+	+

		Undetermined	-	-	-	-	-
		aec11	-	-	-	-	-
		aec14	-	-	-	-	-
		aec15	+	-	-	+	+
		aec16	-	-	-	+	+
		aec17	-	-	-	+	+
		aec18	-	-	-	+	+
		aec19	-	-	-	+	+
		aec22	-	-	-	+	+
		aec23	-	-	-	+	+
		aec24	-	-	-	+	+
		aec25	-	-	-	+	+
		aec26	-	-	-	+	+
		aec27/clpV	-	-	-	+	+
		aec28	-	-	-	+	+
		aec29	-	-	-	+	+
		aec30	-	-	-	+	+
		aec31	-	-	-	+	+
		aec32	-	-	-	+	+
		aec7	-	-	-	-	-
		aec8	-	-	-	-	-
	LEE locus encoded TTSS	*UD1*	-	-	-	-	-
		UD2	-	-	-	-	-
		UD3	-	-	-	-	-
		UD4	-	-	-	-	-
		UD5	-	-	-	-	-
		cesD2	-	-	-	-	-
		cesD	-	-	-	-	-
		cesF	-	-	-	-	-
		cesT	-	-	-	-	-
		escC	-	-	-	-	-
		escD	-	-	-	-	-
		escF	-	-	-	-	-
		escI	-	-	-	-	-
		escJ	-	-	-	-	-
		escK	-	-	-	-	-
		escL	-	-	-	-	-
		escN	-	-	-	-	-
		escO	-	-	-	-	-
		escP	-	-	-	-	-

		escR	-	-	-	-	-
		escS	-	-	-	-	-
		escT	-	-	-	-	-
		escU	-	-	-	-	-
		escV	-	-	-	-	-
		espA	-	-	-	-	-
		espB	-	-	-	-	-
		espD	-	-	-	-	-
		etgA	-	-	-	-	-
		glrA	-	-	-	-	-
		glrR	-	-	-	-	-
		ler	-	-	-	-	-
		sepD	-	-	-	-	-
		sepL	-	-	-	-	-
		sepQ	-	-	-	-	-
	SCI-I T6SS	*UD6*	-	-	-	-	+
		UD7	-	-	-	-	+
		UD8	-	-	-	-	+
		UD9	-	-	-	-	+
		UD10	-	-	-	-	+
		UD11	-	-	-	-	+
		UD12	-	-	-	-	+
		UD13	-	-	-	-	-
		UD14	-	-	-	-	-
		UD15	-	-	-	-	+
		UD16	-	-	-	-	-
		UD17	-	-	-	-	-
		UD18	-	-	-	-	-
		UD19	-	-	-	-	-
		UD20	-	-	-	-	-
		UD21	-	-	-	-	-
		UD22	-	-	-	-	-
		UD23	-	-	-	-	-
		UD24	-	-	-	-	-
		UD25	-	-	-	-	-
		UD26	-	-	-	-	+
		UD27	-	-	-	-	+
		UD28	-	-	-	-	-
		UD29	-	-	-	-	-
		UD30	-	-	-	-	-

		UD31	-	-	-	-	-
		UD32	-	-	-	-	+
Toxin	Alpha-hemolysin	*hlyA*	-	-	-	+	-
		hlyB	-	-	-	+	-
		hlyC	-	-	-	+	-
		hlyD	-	-	-	+	-
	Colicin-like Usp	*usp*	-	-	-	-	-
	Cytolethal distending toxin	*cdtA*	-	-	-	-	-
		cdtB	-	-	-	-	-
		cdtC	-	-	-	-	-
	Cytotoxic necrotizing factor 1	*cnf1*	-	-	-	-	-
	Enterotoxin 1	*set1A*	-	-	-	-	-
		set1B	-	-	-	-	-
	Enterotoxin SenB/TieB	*senB*	-	-	-	-	-
	Heat-labile enterotoxin	*eltA*	-	-	+	+	+
		eltB	-	-	+	+	-
	Heat-stable enterotoxin 1 (EAST1)	*astA*	-	-	-	-	-
	Hemolysin/cytolysin A	*hlyE/clyA*	+	+	+	-	-
	Shiga-like toxin	*stx1A*	-	-	-	-	-
		stx1B	-	-	-	-	-
		stx2A	-	-	-	-	-
		stx2B	-	-	-	-	-

CHAPTER 5: Carbapenem-resistant bacteria over a UWWTP

TABLE S1. General features of the whole genome sequence of isolates.

Feature	**RWM.1**	**RWM.4**	**RWM.8**
Accumulated lenght (bp)	5450047	5518639	5196614
GC content (%)	51.61	51.74	51.84
Number of contigs	149	116	91
N50	91872	179138	191649
Predicted CDS	5567	5561	5115
Predicted contamination[a] (%)	1.63	0.63	0.33
Sequencing technology	Illumina	Illumina	Illumina

[a]According to CheckM tool (Parks et al., 2015).

TABLE S2. Nucleotide identity (ANIb and ANIm) and digital DNA-DNA hybridization (dDDH) calculations based on genome sequences of RWM.4 and RWM.8 against *Citrobacter braakii* ATCC 51113^T (GenBank accession n° NAEW00000000), and of RWM.1 against *Lelliottia jeotgali* PFL01 ^{T} (GenBank accession n° CP018628) and *Lelliottia amnigena* ATCC 33072^T (GenBank accession n° CP015774).

	***C. braakii* ATCC 51113^T (51.9% GC)**		***L. jeotgali* PFL01^T (54.24% GC)**	***L. amnigena* ATCC 33072^T (52.82% GC)**
	RWM.4	**RWM.8**	**RWM.1**	**RWM.1**
% ANIb	98.22	98.32	82.29	81.55
% ANIm	98.96	98.98	84.69	84.42
% ddH	90.50	90.80	25.60	24.80
Dif %GC	0.16	0.07	2.63	1.24

REFERENCES:

Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., Tyson, G.W., 2015. CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 25, 1043–1055. doi:10.1101/gr.186072.114