Universidade de Aveiro Departamento de Biologia
2014

Marta Cristina Oliveira Martins Tacão

Resistência a antibióticos de último recurso em ambientes naturais

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

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… and always look on the bright side of life"
(by Eric Idle in Life of Brian, Monty Python 1979)

palavras-chave

Resistência a antibióticos, ecosistemas aquáticos, beta-lactamases, elementos genéticos móveis

resumo

Antibióticos de último recurso são usados no tratamento de infecções graves causadas por estirpes multiresistentes. A prevalência de bactérias resistentes a estes antibióticos tem aumentado. Os ambientes naturais, influenciados pela actividade humana, são reservatórios de bactérias resistentes e de genes de resistência. Vários genes de resistência com grande impacto na clínica têm presumivelmente origem em estirpes ubíquas em sistemas aquáticos, o que realça a importância destes ambientes na evolução de resistência. Este estudo assenta nas seguintes hipóteses: a) os rios são reservatórios e disseminadores de resistência a antibióticos; b) as atividades antropogénicas potenciam a disseminação de resistência a antibióticos de último recurso nestes ambientes. Assim, foi estabelecido como objectivo comparar o resistoma ambiental referente a antibióticos de último recurso, em rios poluídos e não poluídos. Foram amostrados rios na Bacia Hidrográfica do Vouga, expostos a diferentes impactos antropogénicos. Os rios foram classificados como poluídos e não poluídos de acordo com parâmetros de qualidade da água. Duas colecções foram estabelecidas: bactérias resistentes a cefotaxima (cefalosporina de 3ª geração) e a imipenemo (carbapenemo). Cada colecção foi caracterizada em termos de diversidade filogenética, susceptibilidade a antibióticos, mecanismos de resistência e elementos genéticos móveis. A prevalência de bactérias resistentes foi superior em águas poluídas. Os resultados sugerem que nestes ambientes Enterobacteriaceae, *Pseudomonas* e *Aeromonas* têm um papel importante na disseminação de resistência. Os níveis de resistência a não beta-lactâmicos foram superiores em águas poluídas, assim como o número de estirpes multiresistentes. Detectaram-se genes de beta-lactamases de espectro alargado, associados a elementos genéticos móveis previamente descritos em isolados clínicos. Métodos independentes do cultivo revelaram diferenças claras entre a diversidade de sequências do tipo $bla_{\text{CTX-M}}$ em rios poluídos (idênticas às encontradas em isolados clínicos) e não poluídos (similares a genes ancestrais). A resistência a carbapenemos foi maioritariamente relacionada com a presença de bactérias intrinsecamente resistentes. No entanto, foram identificados genes de carbapenemases relevantes tais como $bla_{\text{OXA-48}}$ em *Shewanella* spp. (origem putativa destes genes) e $bla_{\text{VIM-2}}$ em *Pseudomonas* spp. de rios poluídos. Métodos independentes do cultivo mostraram que, nestes rios, a diversidade de genes similares a $bla_{\text{OXA-48}}$ é superior ao que tem sido relatado. Detectaram-se diferenças evidentes entre rios poluídos e não poluídos, em termos de prevalência, diversidade filogenética e susceptibilidade a antibióticos em bactérias resistentes e ocorrência de genes de resistência clinicamente relevantes. Estes dados validam as hipóteses colocadas. Assim, estes sistemas aquáticos actuam como reservatórios de genes de resistência. As actividades antropogénicas potenciam a disseminação destes genes e a constituição de plataformas genéticas complexas, originando fenótipos de multiresistência que poderão persistir mesmo na ausência de antibióticos.

keywords

Antibiotic resistance, aquatic environments, beta-lactamases, mobile genetic elements

abstract

Last-resort antibiotics are the final line of action for treating serious infections caused by multiresistant strains. Over the years the prevalence of resistant bacteria has been increasing. Natural environments are reservoirs of antibiotic resistance, highly influenced by human-driven activities. The importance of aquatic systems on the evolution of antibiotic resistance is highlighted from the assumption that clinically-relevant resistance genes have originated in strains ubiquitous in these environments. We hypothesize that: a) rivers are reservoirs and disseminators of antibiotic resistance; b) anthropogenic activities potentiate dissemination of resistance to last-resort antibiotics. Hence, the main goal of the work is to compare the last-resort antibiotics resistome, in polluted and unpolluted water. Rivers from the Vouga basin, exposed to different anthropogenic impacts, were sampled. Water quality parameters were determined to classify rivers as unpolluted or polluted. Two bacterial collections were established enclosing bacteria resistant to cefotaxime (3rd generation cephalosporin) and to imipenem (carbapenem). Each collection was characterized regarding: phylogenetic diversity, antibiotic susceptibility, resistance mechanisms and mobile genetic elements. The prevalence of cefotaxime- and imipenem-resistant bacteria was higher in polluted water. Results suggested an important role in the dissemination of antibiotic resistance for Enterobacteriaceae, *Pseudomonas* and *Aeromonas*. The occurrence of bacteria resistant to non-beta-lactams was higher among isolates from polluted water as also the number of multiresistant strains. Among strains resistant to cefotaxime, extended-spectrum beta-lactamase (ESBL) genes were detected (predominantly *bla*$_{\text{CTX-M-like}}$) associated to mobile genetic elements previously described in clinical strains. ESBL-producers were often multiresistant as a result of co-selection mechanisms. Culture-independent methods showed clear differences between *bla*$_{\text{CTX-M-like}}$ sequences found in unpolluted water (similar to ancestral genes) and polluted water (sequences identical to those reported in clinical settings). Carbapenem resistance was mostly related to the presence of intrinsically resistant bacteria. Yet, relevant carbapenemase genes were detected as *bla*$_{\text{OXA-48-like}}$ in *Shewanella* spp. (the putative origin of these genes), and *bla*$_{\text{VIM-2}}$ in *Pseudomonas* spp. isolated from polluted rivers. Culture-independent methods showed an higher than the previously reported diversity of *bla*$_{\text{OXA-48-like}}$ genes in rivers. Overall, clear differences between polluted and unpolluted systems were observed, regarding prevalence, phylogenetic diversity and susceptibility profiles of resistant bacteria and occurrence of clinically relevant antibiotic resistance genes, thus validating our hypotheses. In this way, rivers act as disseminators of resistance genes, and anthropogenic activities potentiate horizontal gene transfer and promote the constitution of genetic platforms that combine several resistance determinants, leading to multiresistance phenotypes that may persist even in the absence of antibiotics.

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GENERAL INTRODUCTION

1.1 ANTIBIOTICS AND ANTIBIOTIC RESISTANCE

An antibiotic can be defined as an organic molecule, of synthetic or natural origin, that inhibits or kills microorganisms by specific interactions with the microbial targets, and that is used for the treatment of infectious diseases in humans and/or other animals (Davies and Davies 2010). Although this definition includes compounds with activity against other microorganisms, the term antibiotics is most often used to define antibacterial substances and will be used in this sense in the context of this thesis. Antibiotics that are used in a clinical context act selectively in central cell processes or structures, distinctive of bacterial cells. Antibiotics can be either bactericidal (induce cell death) or bacteriostatic (inhibit cell growth). Their modes of action include for example the inhibition of processes like peptidoglycan biosynthesis, DNA replication or protein synthesis, or by interfering with the energy metabolism of the cell. The discovery of these substances and their use in medical practice has been one of the major advances of the last century, with great health impact by reducing morbidity and saving countless lives.

On an opposite way, bacterial cells may have the ability to overcome the inhibitory or deleterious effects of antibiotics. In a clinical context, antibiotic resistance leads to prolonged and unsuccessful treatments, augmented costs and ultimately, increased death records (Paul *et al.* 2010, Stokes and Gillings 2011, van Hoek *et al.* 2011). The World Health Organization (WHO) estimates that antimicrobial resistance is the cause of over 15 million deaths per year (WHO 2014a). Infectious diseases remain listed in the 10 leading causes of death in the world (WHO 2014b).

Some key facts recognized by the WHO include: i) infections caused by resistant bacteria fail to respond to conventional treatment, resulting in prolonged illness, greater risk of death and higher costs; ii) a high percentage of hospital-acquired infections are caused by highly resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) or multidrug-resistant Gram-negative bacteria and iii) new resistance mechanisms have emerged, making the latest generation of antibiotics virtually ineffective (WHO 2014a). Hence, the WHO has declared the control of dissemination of antibiotic resistance as one of the top health priorities worldwide (WHO 2014c).

The overuse and misprescription of antibiotics have been pointed out has the main reasons for the increasing of antibiotic resistance. The latest data on the trends of antibiotic consumption in Europe highlights that antibiotics are mostly used outside clinical settings and that intake in the community has been increasing (ECDC 2014).

In Europe, penicillins are the group of antibiotics most frequently used in the community (ECDC 2014). Critical antibiotics as 3rd generation cephalosporins are consumed in higher amounts in hospital settings than in the community (ECDC 2014). The total intake of these antibiotics has increased significantly throughout Europe (ECDC 2014). The treatment of infections caused by bacteria resistant to 3rd generation cephalosporins implies the use of more efficient antibiotics such as carbapenems, which are more expensive and may not be accessible in all clinical settings worldwide (Livermore 2009, Papp-Wallace *et al.* 2011, WHO 2014c). Moreover, the total consumption of carbapenems has also increased in Europe (ECDC 2014).

The recent global report on surveillance of antibiotic resistance presented by WHO (WHO 2014c), emphasizes the high proportion of resistance to extended-spectrum antibiotics, namely 3rd generation cephalosporins, that has been reported worldwide. Furthermore, the same report highlights the upsurge of the proportion of carbapenem-resistant strains among clinically-relevant bacterial groups such as *Acinetobacter* spp., *Pseudomonas* spp. and Enterobacteriaceae, commonly presenting multiresistant traits (resistant to 3 or more classes of antibiotics) and thus limiting the therapeutic options.

Also problematic is the tendency of most pharmaceutical companies to invest in the development of other drugs that are either less regulated and consequently launched faster in the market, or used for long periods of treatment with higher economic retributions (e.g. for the treatment of chronic diseases as diabetes or anti-hypertensive drugs) (Butler *et al.* 2013, Spellberg *et al.* 2004). Thus pharmaceutical companies reduced the investment in antibiotic research and development, since the return on investment in this area was lower.

Antibiotic resistance is a multifactorial problem (FIG. 1). Besides the abusive use and misuse of antibiotics in human and veterinary medicine, several other aspects can contribute to the emergence and spread of antibiotic resistance.

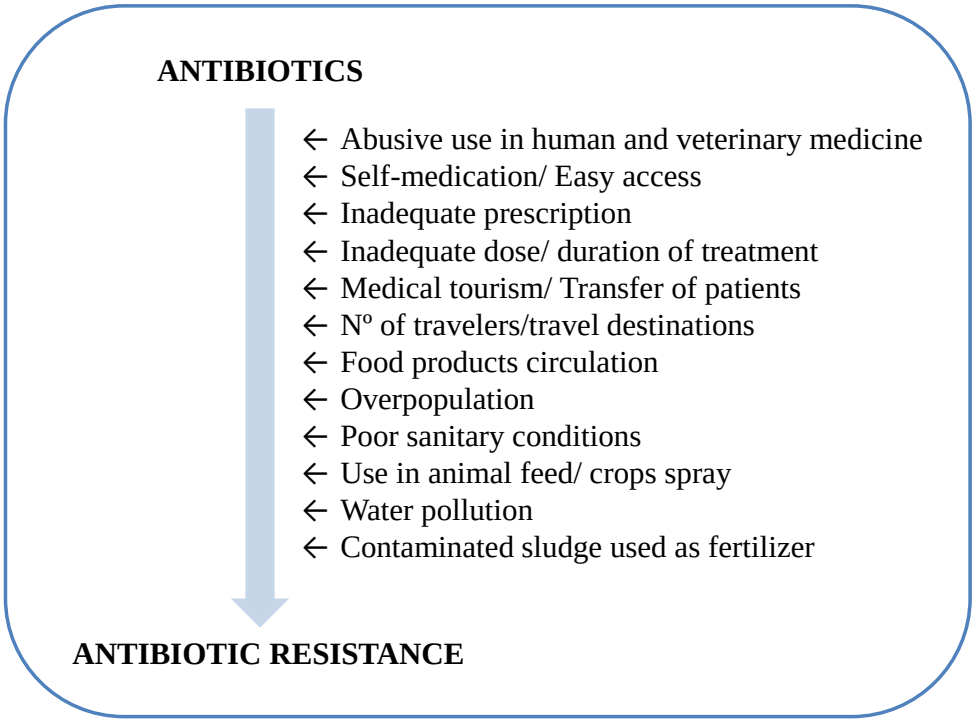

FIG.1: Factors influencing the increasing emergence of antibiotic resistance.

Over the years numerous changes have occurred on a global scale and in diverse segments that contributed for the increase of resistance levels registered worldwide. From a social perspective, migration and population growth added for the widespread of antibiotic resistance. Countries that are overpopulated, and consequently have in general poor hygiene and sanitary conditions, are dealing with serious infections caused by the most problematic multiresistant strains (Nordmann *et al.* 2011, Poirel *et al.* 2012a). The transfer of patients from these to other countries contributes to the spread of antibiotic resistance (Poirel *et al.* 2012a). The intensification of traveling events, the increasing number of travelers and medical tourism enhance this problem too (Rogers *et al.* 2011). Other factors that have been also contributing for this problematic scenario are the new commercial routes that promote the worldwide distribution of a variety of food products (Cabello *et al.* 2013, Durso and Cook 2014). Finally the abusive use of antibiotics in farms and aquacultures as food additives to promote animal growth and/or to prevent diseases contribute for the increasing prevalence of antibiotic resistance (Cabello *et al.* 2013, Durso and Cook 2014, Rolain 2013).

Alexander Fleming, who discovered penicillin, in his 1945 Nobel Lecture alerted to the consequences of the abusive use of antibiotics and foretold "*the time may come when penicillin can be bought by anyone in the shops. Then, there is the danger that the ignorant man may easily under-dose himself and by exposing his microbes to non-lethal-quantities of the drug educate them to resist*" (Fleming 1945). In fact, by this time it had already been identified a bacterial penicillinase, conferring resistance to penicillins (Abraham and Chain 1940).

In the above statement, Fleming also acknowledged the importance of antimicrobial stewardship, and emphasized other two important aspects that still contribute to the emergence and prevalence of antimicrobial resistance nowadays: self-medication and drug regimen disregard (both dose and duration of treatment).

If we look at the antibiotic resistance timeline, that is the time between the introduction of an antibiotic in clinical practice and the first report of antibiotic resistance towards it (FIG. 2), we observe that for some antibiotics the emergence of resistance is quite fast, since in few years the antibiotic therapeutic potential is compromised. From the beginning of the antibiotic era we have been witnessing a successional chain of events that start with the inclusion of a new antibiotic followed by the emergence of resistant organisms and in a next step a new antibiotic is launched to deal with the resistant bacteria. This cycle is constantly repeated, although the disinvestment in the development of new drugs slowed it down in recent years.

In 2008, L.B. Rice recognized as most worrisome pathogens, increasingly prevalent and multiresistant, both Gram-positive and Gram-negative bacteria that he defined as the (no) ESKAPE bugs. These include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species (Rice 2008).

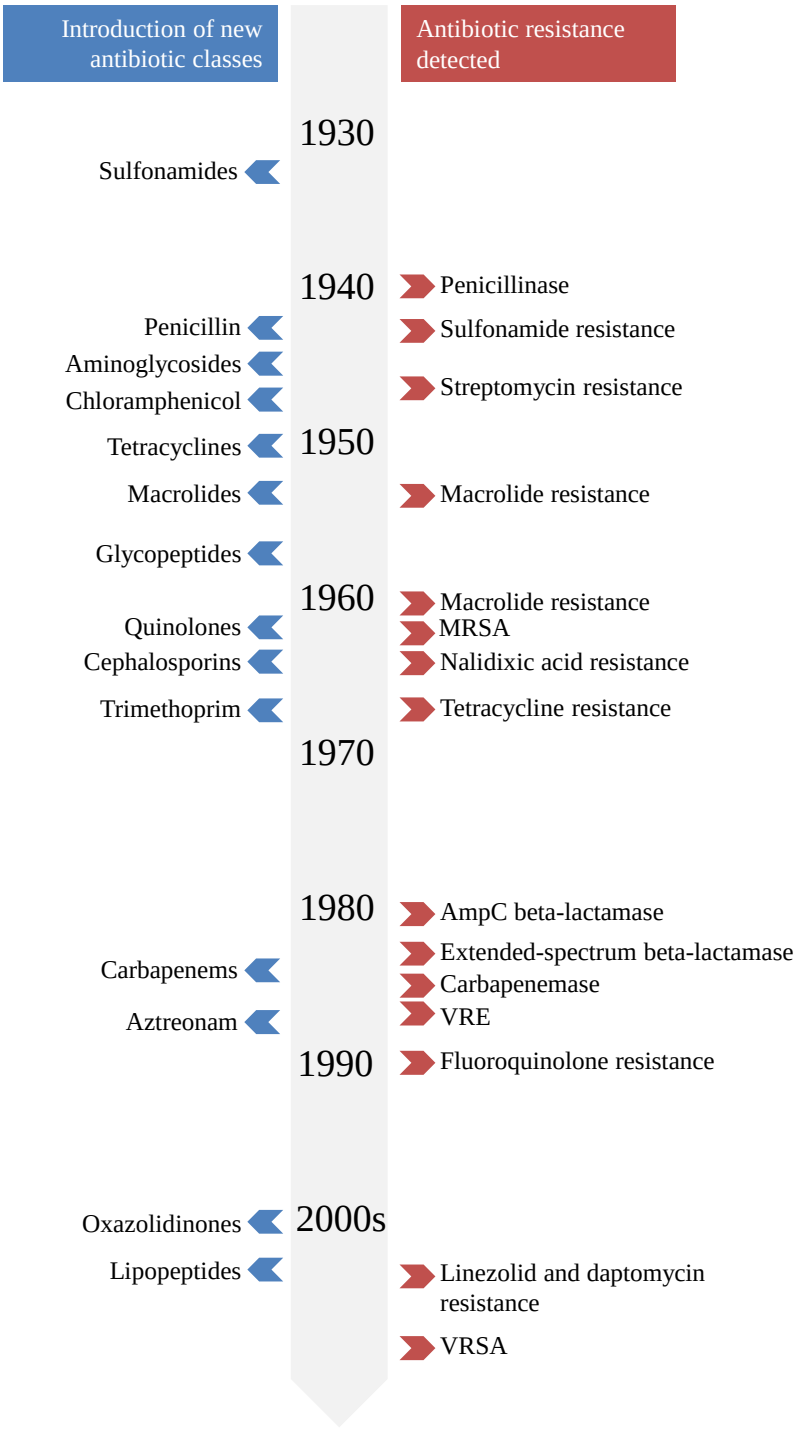

FIG. 2: Timeline displaying the introduction of some of the antibiotics commonly used in clinical settings and the first report of resistance to those antibiotics. MRSA - methicillin-resistant *Staphylococcus aureus*, VRE - vancomycin-resistant *Enterococcus*; VRSA - vancomycin-resistant *Staphylococcus aureus* (adapted from CDC 2013).

Although there has been a decline on the discovery and development of new antimicrobials, there are a number of rather new drugs still available to treat infections caused by Gram-positive bacteria, such as ceftaroline, daptomycin, linezolid and tigecycline (Bush 2010, Kauffman 2003, Patterson 2000, Steenbergen *et al.* 2005, Zhanel *et al.* 2009). In opposition, drug development for the treatment of infections caused by Gram-negative bacteria has stagnated and no new class of antibiotics has been proposed for over 50 years (Bush 2012). Noteworthy, 4 out of 6 of the ESKAPE bugs are Enterobacteriaceae or non-fermenters Gram-negatives. Thus the study of resistance to antibiotics that are critically important to treat infections caused by Gram-negative bacteria is of maximum priority.

Mostly, new antibiotics offered to treat infections caused by Gram-negative bacteria are analogues of former existing drugs with improved and/or broader spectrum of activity or new combinational therapies such as beta-lactam/beta-lactamase inhibitors (Bush 2012, Butler *et al.* 2013, Page and Heim 2009, Silver 2011). Recently, a potent metallo-beta-lactamase inhibitor was identified in a strain of *Aspergillus versicolor*, named aspergillomarasmine A (AMA). This fungal natural product when combined with meropenem allowed to fully restore the activity of the antibiotic, against Enterobacteriaceae members, *Acinetobacter* spp. and *Pseudomonas* spp. producing the clinically-relevant NDM-1 or VIM-2 enzymes. These enzymes confer resistance to meropenem and other carbapenems and present a broad range of hydrolytic activity (King *et al.* 2014).

Additionally, older drugs such as colistin, previously rejected due to their toxic properties, are currently being used in the absence of therapeutic alternatives for treatment of severe infections caused by highly resistant Gram-negative pathogens (Falagas *et al.* 2005).

1.2 ANTIBIOTIC RESISTANCE MECHANISMS AND DISSEMINATION

Several antibiotics are produced by naturally-occurring microorganisms most probably to inhibit competitors, and thus, it would be expected that antibiotic resistance mechanisms in nearby microorganisms would have the purpose of escaping antibiotics action (Baquero *et al.* 2009, Davies and Davies 2010, D'Costa *et al.* 2011).

Years before the beginning of the antibiotic era, that is, when large-scale production and introduction of antibiotics into medical practice started, antibiotic resistance had already been acknowledged. Actually, prior to the use of penicillin in medical practice, the first natural antibiotic discovered, a bacterial penicillinase was identified (Abraham and Chain 1940). More recently, studies developed using ancient DNA from archeological findings proved that antibiotic resistance genes were present in the bacterial flora of humans at least 1000 years before the start of the antibiotic era (Appelt *et al.* 2014, Warinner *et al.* 2014). Also it has been discussed that currently known genetic determinants of resistance presented originally other functions in the cell (including antibiotic biosynthesis), that later turned useful for dealing with these drugs (Baquero *et al.* 2009, Martinez 2009a).

Resistance mechanisms include target substitution and modification, membrane permeability alterations, production of enzymes that inactivate the antibiotic and efflux pumps to expel the antibiotic from the cell or reduce its concentration below an efficient level. Table 1 shows the most commonly used antibiotics for the treatment of infections caused by Gram-negatives, their mode of action/target and resistance mechanisms.

TABLE 1: Common resistance mechanisms and modes of action in most used antibiotics for the treatment of infections caused by Gram-negative bacteria.

Antibiotic class	Examples	Mode of action (target)	Resistance mechanisms
Beta-lactams	penicillins (ampicillin, amoxicillin), cephalosporins (cefotaxime, ceftazidime), carbapenems (imipenem, ertapenem, meropenem), monobactam (aztreonam)	Inhibit the synthesis of the bacterial cell wall (peptidoglycan biosynthesis)	Hydrolysis, efflux pumps, target modification, loss or alteration in outer membrane porins
Quinolones	ciprofloxacin nalidixic acid	Interact with the synthesis of DNA (DNA replication)	Acetylation, efflux pumps, target modification
Aminoglycosides	kanamycin gentamicin	Inhibit protein synthesis (translation)	Phosphorylation, acetylation, efflux pumps, target modification
Sulfonamides	sulfonamide	Modify the energy metabolism of the cell (C1 metabolism)	Hydrolysis, efflux pumps, target modification
Phenicols	chloramphenicol	Inhibit protein synthesis (translation)	Acetylation, efflux pumps, target modification
Tetracyclines	tetracycline tigecycline	Inhibit protein synthesis (translation)	Monooxygenation, efflux pumps, target modification
Pyrimidines	trimethoprim	Modify the energy metabolism of the cell (C1 metabolism)	Efflux pumps, target modification

Besides the traditionally referred antibiotic resistance mechanisms, bacterial communities have developed other strategies for overcoming the antibiotics action. For example it has been shown that the formation of biofilms increases the bacterial ability to survive in the presence of these compounds (Høiby *et al.* 2010). Biofilms are particularly problematic when associated for example to medical implants (Høiby *et al.* 2010, Mah and Toole 2001). It has been reported that bacterial cells when in community, attached to a solid surface and embedded in an exopolysaccharide matrix can become 10–1000 times

more resistant to the effects of antimicrobial drugs (Mah and Toole 2001). The matrix provides protection and security against not only antibiotics but also against the immune and inflammatory responses of the host (Høiby *et al.* 2010). Another example of mechanism contributing to increase resistance levels is the heteroresistance phenomenon that has been already associated to antibiotic treatment failures (Wang *et al.* 2014). Heteroresistance is observed when within a clonal population there are sub-populations of antibiotic-resistant and antibiotic-sensitive cells. It has already been described in some clinically-relevant microorganisms as for example *S. aureus* (Nunes *et al.* 2006, Rinder *et al.* 2001, Ryffel *et al.* 1994), *A. baumannii* (Hung *et al.* 2012) or *K. pneumoniae* (Tato *et al.* 2010).

Resistance to an antibiotic is a characteristic that can either be inherent or acquired. Intrinsic features can be the expression of genes encoding hydrolyzing enzymes: for example, bla_{CphA} in some *Aeromonas* spp. or bla_{L1} in *Stenotrophomonas maltophilia*, coding respectively for the CphA and L1 metallo-beta-lactamases, which confer resistance to carbapenems (Avison *et al.* 2001, Walsh *et al.* 2005). Another intrinsic characteristic is the impermeability of the outer membrane in Gram-negative bacteria towards many molecules such as macrolides (Cox and Wright 2013). Intrinsic resistance may also be mediated by active efflux pumps that decrease the intracellular concentration of the antibiotic. Examples of this later resistance mechanism are the multidrug-efflux pumps chromosomally-encoded in *P. aeruginosa*, which confer resistance to at least 3 classes of antibiotics: beta-lactams, fluoroquinolones and aminoglycosides (Cox and Wright 2013, Livermore 2001, Mesaros *et al.* 2007, Strateva and Yordanov 2009).

On the other hand, to build resistance the main genetic mechanisms are mutation, and horizontal gene transfer (HGT). The rate by which a resistant microorganism appears is determined by the combined frequency of "*de novo*" mutation within the bacterial genome and lateral transfer events (Andersson and Hughes 2010).

There are three main processes that promote horizontal gene transfer: conjugation (cell-to-cell transfer), transformation (DNA-to-cell transfer) and transduction (phage-mediated transfer). These mechanisms involve the mobilization of diverse genetic platforms such as plasmids, transposons and integrons, all of which play an important role on the spread of resistance to antibiotics but also of resistance towards other compounds

such as heavy metals (Carattoli 2013, Rodriguez-Rojas *et al.* 2013). For centuries, heavy metals were used for the treatment of several diseases before the use of antibiotics in clinical settings and this practice may have contributed for the selection of genetic platforms encoding both heavy metals and antibiotic resistance (Baker-Austin *et al.* 2006). By capturing one single mobile element, one microorganism can acquire multiresistant traits to a wide range of compounds.

Also to take into account when discussing antibiotic resistance dissemination is the fact that there are highly effective strains, that is, strains that are quite successful on spreading genetic determinants of resistance both vertical and horizontally, with great propensity to acquire foreign genes. Hence, these high risk clones show a great epidemiological success, being found widely distributed (Woodford *et al.* 2011). Examples include the ST131 *Escherichia coli* clone that usually carries a $bla_{\text{CTX-M}}$ (Nicolas-Chanoine *et al.* 2008) and the ST258 *K. pneumoniae* with bla_{KPC} (Kitchel *et al.* 2009). These multidrug resistant clones that have been identified in multiple locations (Woodford *et al.* 2011). $bla_{\text{CTX-M}}$ genes are a paradigmatic example of success in terms of dissemination (Cantón *et al.* 2012, Davies and Davies 2010). Their huge success is due not only to their association to genetic platforms responsible for their mobilization and dissemination (insertion sequences, integrons, transposons, plasmids), but also to the fact that these platforms might be carried by multiple successful clones (Cantón and Coque 2006) (FIG. 3).

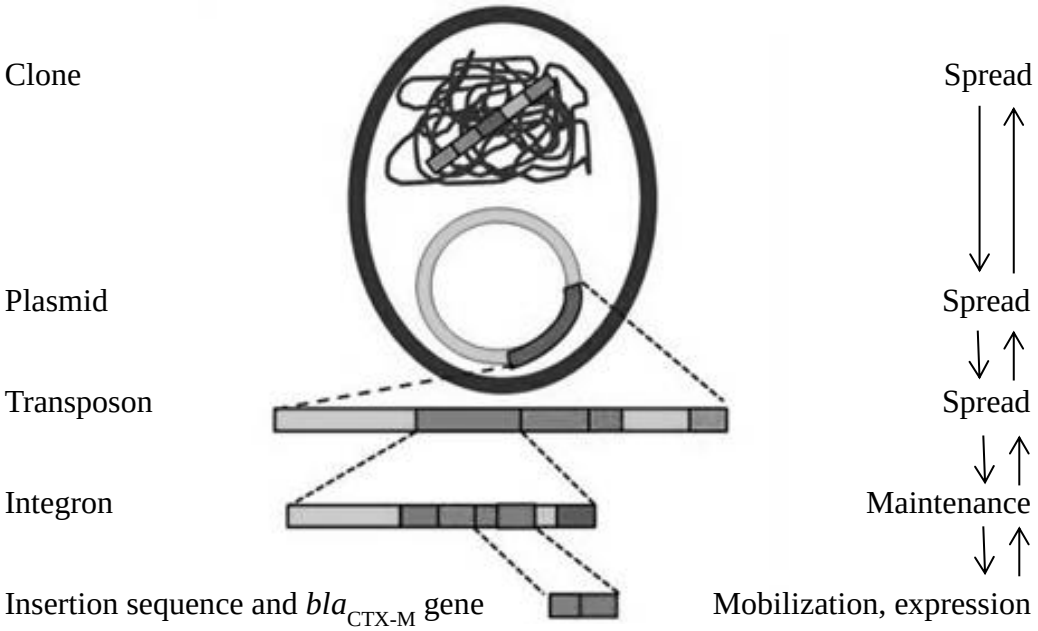

FIG.3: Spread and mobilization of $bla_{CTX\text{-}M\text{-}like}$ genes (Cantón *et al.* 2012).

1.3 BETA-LACTAMS AND BETA-LACTAMASES

Due to their high efficiency and low toxicity for the host, beta lactams are the most widely used antibiotics for the treatment of infections caused by Gram-negative bacteria (Bush 1999). Beta-lactams act by inhibiting the peptidoglycan synthesis and contain a beta-lactam ring in their chemical structure. The most common mechanism of resistance to these antibiotics in Gram-negative bacteria consists in the production of beta-lactamases, which are enzymes that hydrolyze the amide bond of the beta-lactam ring and by doing so, inactivate the antibiotic. Table 2 presents relevant beta-lactamase families, with their classification according to their functional (Bush-Jacoby groups) (Bush *et al.* 1995) and molecular characteristics (Ambler classes) (Ambler 1980), the hydrolytic spectrum, current approximate number and representative enzymes.

1.3.1 Last-resort beta-lactams

At the turn of the century, the resistance mechanisms that put in peril the use of most successful and potent beta-lactam antibiotics were identified as major threats for the 21st century, from a clinical viewpoint (Bush 1999). These mechanisms confer resistance to antibiotics referred nowadays as last-resort antibiotics, that is, the last therapeutic options for serious bacterial infections. Carbapenems are last-resort antibiotics for the treatment of a wide range of serious infections caused by gram-negative bacteria. Extended-spectrum cephalosporins (3rd and 4th generation) are frequently used in hospitals to treat serious life-threatening diseases. These are also considered last-resort antibiotics in specific cases as for example for the treatment of gonorrhea and some types of meningitis (WHO 2014).

Third generation cephalosporins and carbapenems were first introduced in medical practice during the 80's. The Food and Drug Administration (FDA) approved the first 3rd generation cephalosporin early in the 80s, the cefotaxime (Todd and Brogden 1990), and in 1985 the first carbapenems (imipenem) for the treatment of serious infections (Papp-Wallace *et al.* 2011). Short after their introduction in medical practices resistance mechanisms were detected.

TABLE 2: Beta-lactamase families with clinical relevance (PEN-penicillins; Ecep- early cephalosporins; Bcep- broad-spectrum cephalosporins; CAR-carbapenems; MON - monobactams).

Enzyme family	Molecular class	Functional group	Inhibited by		Representative enzymes	Nº of enzymes*	Hydrolytic substrate spectrum				
			CA or TZB	EDTA			PEN	ECep	BCep	CAR	MON
AmpC/ CMY-like	C	1	No	No	CMY-1 to CMY-120, ACC-1 to ACC-6, ACT-1 to ACT-34, DHA-1 to DHA-22, FOX-1 to FOX-12, MIR-1 to MIR-15, MOX-1 to MOX-9	over 200					
TEM-1 SHV-1	A	2b	Yes	No	TEM-1, TEM-2 SHV-1	over 15 over 30					
TEM	A	2be	Yes	No	TEM-3 to TEM-12 TEM-15 to TEM-29 TEM-130, TEM-211	over 80					
SHV	A	2be	Yes	No	SHV-2 to SHV-9 SHV-45, SHV-55 SHV-70, SHV-90	over 45					
CTX-M VEB PER	A	2be	Yes	No	CTX-M-1 to CTX-M-152 VEB-1 to VEB-9 PER-1 to PER-7	152 9 7					
GES	A	2be	Yes	No	GES-1, GES-9, GES-11	5					
OXA-ESBLs	D	2de	variable	No	OXA-11, OXA-14, OXA-15, OXA-16 OXA-28, OXA-35	over 20					
IMI KPC GES SME	A	2f	variable	No	IMI-1 to IMI-5 KPC-2 to KPC-18 GES-2 to GES-6, GES-14 SME-1 to SME-5	5 17 10 5					
IMP VIM NDM IND	B	3a	No	Yes	IMP-1 to IMP-48 VIM-1 to VIM-41 NDM-1 to NDM-10 IND-1 to IND-15	48 41 10 15					
OXA-Carbap.	D	2df	variable	No	OXA-23, OXA-48, OXA-58, OXA-181, OXA-199, OXA-204, OXA-232, OXA-162, OXA-163	over 50					

* www.lahey.org/studies/; last accession June 2014

The hydrolysis of these antibiotics by enzymes as extended-spectrum beta-lactamases (ESBLs) and carbapenemases is the most common bacterial resistance mechanism. While ESBLs hydrolyze penicillins, cephalosporins and monobactams, carbapenemases are diverse in terms of resulting phenotype but some might neutralize all beta-lactams.

1.3.1.1 Resistance to 3rd generation cephalosporins

In Gram-negative bacteria resistance to 3rd generation cephalosporins is mainly attributed to: i) high-level expression of an intrinsic *ampC* gene (through mutations in the promoter region) ii) plasmid-encoded *ampC* genes and iii) production of extended-spectrum beta-lactamases (ESBLs) (Patel and Bonomo 2013, Pfeifer *et al.* 2010).

1.3.1.1.1 AmpC and extended-spectrum beta-lactamases

AmpC cephalosporinases are included in Ambler class C and Bush-Jacoby functional group 1. The first beta-lactamase described was in fact an AmpC beta-lactamase, identified in an *E. coli* isolate (Abraham and Chain 1940). AmpC cephalosporinases expression is inducible by certain beta-lactams as ampicillin and clavulanic acid (Jacoby 2009). Furthermore hyperproduction of these enzymes can convey resistance also to carbapenems, even if the bacteria lack other resistance mechanisms (Harris and Ferguson 2012, Patel and Bonomo 2013, Pfeifer *et al.* 2010). In this way, the hydrolytic spectrum of activity includes penicillins and early and extended spectrum cephalosporins, but also carbapenems if induced.

AmpC beta-lactamases are commonly found in the chromosome of Enterobacteriaceae and also *Pseudomonas* spp. Although less frequent, AmpC cephalosporinases have also been detected in plasmids. Most of those plasmid-encoded genes, like ACC, ACT, DHA, FOX, MOX or the most widespread CMY, seem to be derived from chromosomal variants

(Table 2). Moreover, isolates that present plasmidic AmpC enzymes usually produce other penicillinases or cephalosporinases (Jacoby 2009).

Extended-spectrum beta-lactamases (ESBLs) are included in Ambler class A and Bush-Jacoby functional group 2be. These beta-lactamases encompass in their hydrolytic spectrum penicillins, early and extended-spectrum cephalosporins, monobactams but not carbapenems. For a long time, the most prevalent ESBLs detected in clinical Gram-negative bacteria were variants of SHV and TEM families that by single or multiple mutations on initial SHV-1 and TEM-1 penicillinases, had expanded their hydrolytic spectrum to enclose also extended-spectrum cephalosporins and monobactams (Bush *et al.* 1995, Bush 2010, Paterson and Bonomo 2005). Over the last decade CTX-M-type prevalence increased and rapidly became the most commonly reported ESBL (Cantón and Coque 2006, Livermore *et al.* 2007, Perez *et al.* 2007). In fact, its fast dissemination has been referred by some authors as the "CTX-M pandemic" (Cantón and Coque 2008) (FIG. 4). As stated previously, the increasing number of $bla_{\text{CTX-M-like}}$ genes detected worldwide is due mainly to efficient mobilization promoted by highly successful clones (Cantón and Coque 2008, Cantón *et al.* 2012, Davies and Davies 2010, Woodford *et al.* 2011).

The association of $bla_{\text{CTX-M-like}}$ genes to mobilizable genetic structures contributes to the maintenance of ESBL-producing strains under different selective pressures since most carry other genetic determinants that encode resistance to other compounds or classes of antibiotics. In fact antibiotic multiresistant traits among ESBL-producers are common. Usually, these strains present co-resistance to aminoglycosides, quinolones and tetracyclines (Coque *et al.* 2008, Cantón *et al.* 2012, Perez *et al.* 2007).

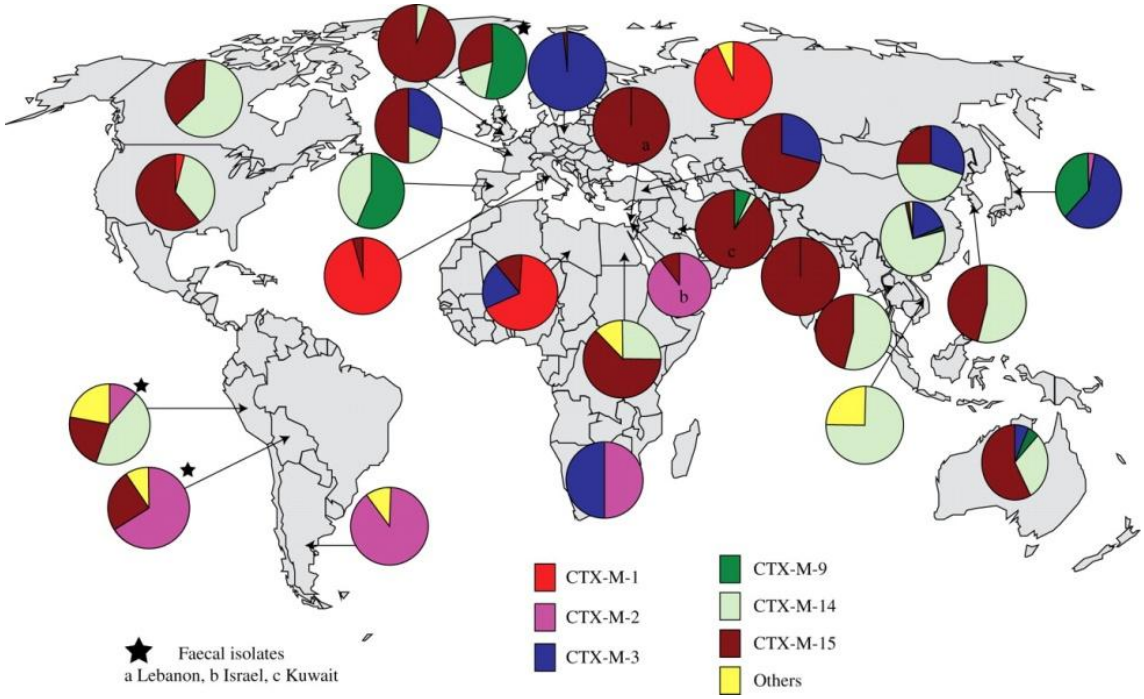

FIG.4: Global distribution of different families of CTX-M beta-lactamases (Davies and Davies 2010).

Different genetic elements have been associated to *bla*$_{\text{CTX-M}}$ genes. Particularly common on the genomic environment of these genes are insertion sequences such as ISEcp1 and IS26 (Bush and Fisher 2011, Cantón and Coque 2006, Coque *et al.* 2008). Also *bla*$_{\text{CTX-M}}$ genes have been linked to both narrow- and broad-host range plasmids, belonging to IncA/C, IncF, IncHI2, IncI1, IncK, IncL/M and IncN groups, that often carry other antibiotic resistance genes (Cantón and Coque 2006; Carattoli 2009, Carattoli 2011). There are over 150 CTX-M-like ESBLs described so far (www.lahey.org/studies/; last accession June 2014), and mostly were found in clinical Enterobacteriaceae, but also *Pseudomonas* spp., *Acinetobacter* spp, *Vibrio* spp. and *Aeromonas* spp. (Cantón *et al.* 2012, Chen *et al.* 2010, Coque *et al.* 2008, Novais *et al.* 2010, Picão *et al.* 2009, Woodford *et al.* 2011). As stated before, the association of *bla*$_{\text{CTX-M}}$ genes to successful clones has contributed to their rapid dissemination, as for example the *E. coli* ST131 clone mainly responsible for the worldwide spread of the *bla*$_{\text{CTX-M-15}}$ gene (Nicolas-Chanoine *et al.* 2008, Poirel *et al.* 2012c, Rogers *et al.* 2011a).

Although TEM, SHV or CTX-M variants are prevalent, other unrelated class A ESBL families have been detected such as GES, PER, VEB, BES, BEL and TLA types (Naas *et al.* 2008, Poirel *et al.* 2012c). Whereas some are still regionally constrained (e.g. BES, BEL and TLA types) and rarely detected (Naas *et al.* 2008), others have spread in severall continents (e.g. PER and VEB types). PER-like ESBLs have been identified mostly in *P. aeruginosa* and *Acinetobacter* spp. but also in Enterobacteriaceae members (Naas *et al.* 2008), *Aeromonas caviae* (Girlich *et al.* 2010b, Maravić *et al.* 2013) and *Vibrio cholerae* (Petroni *et al.* 2002). VEB-like ESBLs have been identified also in *Acinetobacter* spp., *P. aeruginosa* and Enterobacteriaceae members (Naas *et al.* 2008). Co-resistance to quinolones and extended-spectrum beta-lactams is frequently reported in VEB-like-producers (2005b). GES-like ESBLS vary in their hydrolysis profile as unlike most ESBLs some do not hydrolyze monobactams (Table 2). These have been characterized in clinical *Pseudomonas* spp., *A. baumannii* and Enterobacteriaceae members (Poirel *et al.* 2012c), but also in environmental *Aeromonas* spp. (Girlich *et al.* 2011).

Due to the high homology with chromosomal beta-lactamase genes of the non-clinical genus *Kluyvera* (Poirel *et al.* 2002), the CTX-M-like ESBLs are thought to have KLUC from *Kluyvera cryocrescens* as ancestor of CTX-M-1 (Decousser *et al.* 2001), KLUA from *Kluyvera ascorbata* of CTX-M-2 (Humeniuk *et al.* 2002), KLUG from *Kluyvera georgiana* of CTX-M-8 (Poirel *et al.* 2002) and KLUY from *K. georgiana* of CTX-M-9 (Olson *et al.* 2005). Several other chromosomal class A ESBLs have been described: examples are RAHN-1 and RAHN-2 in *Rahnella* spp. (Bellais *et al.* 2001, Ruimy *et al.* 2010), and FONA in *Serratia fonticola,* which is the putative progenitor of SFO-1 enzyme (Peduzzi *et al.* 1997).

Besides class A ESBLs, there are also class D enzymes often referred as OXA-ESBLs. These beta-lactamases are poorly inhibited by clavulanic acid and weakly hydrolyze broad-spectrum cephalosporins (Patel and Bonomo 2013). OXA-ESBLs are mostly prevalent in non-fermenters as *Pseudomonas* spp. and *Acinetobacter* spp. (Bush and Fisher 2011, Evans and Amyes 2014, Patel and Bonomo 2013).

As the occurrence of infections caused by AmpC/ESBL-producing bacteria continues rising, with the majority presenting a multiresistant phenotype, treatment options are decreasing and so the use of carbapenems is more frequent (Livermore 2009).

1.3.1.2 Resistance to carbapenems

Resistance towards carbapenems is often mediated by intrinsic or acquired carbapenemases. But also, as for example in some *Pseudomonas spp.,* carbapenem resistance results from the concerted action of high level expression of AmpC cephalosporinases, non-enzymatic mechanisms such as reduced outer membrane permeability and overexpression of efflux pumps (Harris and Ferguson 2012, Livermore 2001, Mesaros *et al.* 2007, Strateva and Yordanov 2009).

Over the last 10 to 15 years the prevalence of carbapenem-resistant Gram-negative bacteria has been increasing worldwide, largely related to the production and spread of carbapenemases (Nordmann *et al.* 2011, Queenan and Bush 2007). While some are still geographically constrained, others have spread on a much wider scale (Patel and Bonomo 2013). Moreover, the carbapenemases epidemiology is particularly worrying in countries facing serious outbreaks like for example the Indian subcontinent with NDM carbapenemases or USA and Greece with KPC carbapenemases (Nordmann and Poirel 2014). In Europe, carbapenem resistance among clinically-relevant Enterobacteriaceae has increased during the last decade but there are still few countries where only sporadic cases have been reported (FIG. 5) (ECDC 2013a, Glasner *et al.* 2013).

Carbapenemases diverge in terms of host diversity, enzyme activity and substrate specificity, varying from narrow to extended ranges (Bush 2013, Cornaglia *et al.* 2011, Nordmann *et al.* 2011). Carbapenemases include metallo-beta-lactamases with one or two zinc ions on the active site (Ambler class B) and serine carbapenemases with serine at the active site (Ambler classes A and D) (Queenan and Bush 2007).

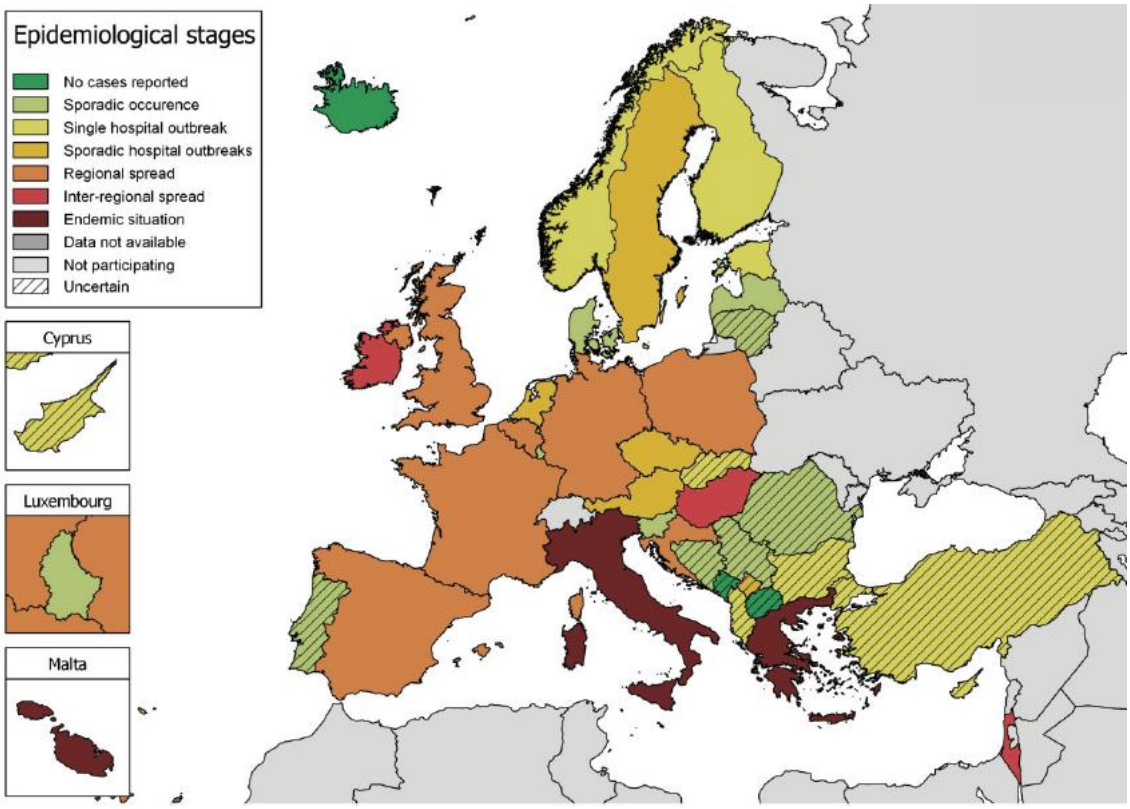

FIG. 5: Occurrence of carbapenemase-producing Enterobacteriaceae in 38 European countries based on self-assessment by the national experts, March 2013 (ECDC 2013a).

Until the recognition of the first plasmid-encoded carbapenemases (e.g. IMP-1 in *P. aeruginosa* and OXA-23 in *A. baumannii*), it was though that all carbapenemases identified were chromosomally-encoded and species-specific (Queenan and Bush 2007). Examples of chromosomally-encoded carbapenemases include the class A SME-1 first identified in a *Serratia marcescens* isolate (Naas *et al.* 1994), IMI and NMC-A in clinical *Enterobacter cloacae* isolates (Nordmann *et al.* 1993, Rasmussen *et al.* 1996), SFC-1 in a *S. fonticola* strain (Henriques *et al.* 2004), but also class B carbapenemase CphA in *Aeromonas* spp. (Massidaa *et al.* 1991, Walsh *et al.* 2005) and Sfh-I in *S. fonticola* (Saavedra *et al.* 2003).

Strains of Gram-negative bacteria that acquire carbapenemases by horizontal gene transfer pose an extra concern when they are the cause of infections: carbapenemases carried by mobile genetic elements are often associated to other resistance determinants

(Bush 2013, Patel and Bonomo 2013). This results in strains displaying multiresistance traits, as is the case of bacteria carrying plasmid-encoded ESBLs.

1.3.1.2.1 Metallo-beta-lactamases

All metallo-enzymes show strong carbapenemase activity, are inhibited by monobactams but not by beta-lactamase inhibitors. Although these enzymes do not hydrolyze monobactams as aztreonam, hosts often co-produce an ESBL which has the ability to inactivate these antibiotics (Bush 2010, Cornaglia *et al.* 2011).

The first plasmid-encoded metallo-beta-lactamases (IMP-1) was reported in Japan in the early 1990s (Ito *et al.* 1995). Although it was expected a rapid dispersal of these enzymes, only several years later metallo-beta-lactamases observations increased as carbapenems use was promoted by the augmented prevalence of infections caused by ESBL-producers. Currently, the most relevant enzyme families in terms of medical importance are those belonging to the IMP-, VIM- and most recently the NDM-families (Patel and Bonomo 2013).

As has occurred with other beta-lactamases, initially metallo-enzymes were geographically constrained but nowadays the majority of these carbapenemases have been detected worldwide, mostly in Enterobacteriaceae and non-fermenters as *Pseudomonas* spp. and *Acinetobacter* spp. (Cornaglia *et al.* 2011, Nordmann *et al.* 20011, Patel and Bonomo 2013, Walsh *et al.* 2005).

bla$_{VIM}$ and *bla*$_{IMP}$ are often present as gene cassettes in class 1 integrons (http://integrall.bio.ua.pt; Moura *et al.* 2009). These genetic platforms might accumulate genes encoding resistance towards other classes of antibiotics or even other compounds, conferring an extra advantage to their hosts. IMP and VIM were the most frequently detected metallo-enzymes, of which IMP-1 and VIM-2 are the most prevalent. Presently, there are 48 and 41 variants described of VIM and IMP, respectively (www.lahey.org/studies/; last accession June 2014) that have been described mostly in

clinically relevant Gram-negatives as *Pseudomonas* spp., *Acinetobacter* spp. and Enterobacteriaceae members (Nordmann *et al*. 2011).

NDM-1 was first identified in a *K. pneumoniae* isolate in 2008, from a patient who had recently traveled from India (Yong *et al.* 2009). In fact, following studies performed with isolates collected years before NDM first report showed that most probably this enzyme had been circulating in India long before its first observation (Castanheira *et al.* 2011). Moreover, the first NDM-related cases reported epidemiological links to that country, thus, international travel and medical tourism were pointed has main causes for its dispersion (Johnson and Woodford 2013, Patel and Bonomo 2013). Nowadays it has been detected in all continents and there are 10 variants described (www.lahey.org/studies/; last accession June 2014) but still NDM-1 is predominant (Nordmann *et al.* 2011b). Due to their association to a wide range of hosts (*Acinetobacter* spp., *Aeromonas* spp., *V. cholerae*, *Stenotrophomonas* spp., Enterobacteriaceae members) and different plasmids (IncA/C, IncL/M, IncF), it is expected NDM-producers to become commonly isolated (Nordmann *et al.* 2011b). No dominant clone among bla_{NDM}-carrying isolates has been identified, in contrast with what was observed for other carbapenemase genes (Nordmann *et al.* 2011, Nordmann *et al.* 2011b). Far more disturbing is the fact that, in similar way as other carbapenemase- and ESBL-producers, also NDM-carrying isolates present multiresistance traits, carrying genetic determinants of resistance to other classes of antibiotics as for example, aminoglycosides, quinolones or tetracyclines (Nordmann *et al.* 2011a, Nordmann *et al.* 2011b).

Most frequently, metallo-enzymes are identified together with other beta-lactamases, usually TEM-1, as also SHV and CTX-M enzymes, and CMY-like cephalosporinases. By producing multiple beta-lactamases, even though sometimes with coinciding substrate profiles, these strains are resistant to all beta-lactams. For example, a clinical *K. pneumoniae* strain isolated in Greece co-produced TEM-1, CMY-2, CTX-M-15, VIM-19 and KPC-2 (Pournaras *et al.* 2010). Examples of carbapenem-intrinsically-resistant strains that carry additional beta-lactamases are the *S. maltophilia* strains that co-produce the L1 carbapenemase and the L2 cephalosporinase (Avison *et al.* 2001) as also *Aeromonas hydrophila* strains that co-produce the carbapenemase CphA, but also the penicillinase ampH and the cephalosporinase cepH (Massidda *et al.* 1991, Walsh *et al.* 1997).

1.3.1.2.2 Serine carbapenemases

Serine carbapenemases include both class A and class D enzymes, varying also in host range and distribution. Chromosomally-encoded class A serine carbapenemases are rarely isolated and geographically restrained. These include the NMC-A identified so far only in clinical *E. cloacae* isolates (Nordmann *et al.* 1993), SME-1 and SME-2 in *S. marcescens* isolates (Naas *et al.* 1994), SFC-1 in an environmental isolate of *S. fonticola* (Henriques *et al.* 2004), BIC-1 in an environmental *Pseudomonas fluorescens* strain (Girlich *et al.* 2010) and IMI-1 in *E. cloacae* (Rasmussen *et al.* 1996).

The KPC family of enzymes is the most clinically-relevant group of class A carbapenemases. These plasmid-encoded enzymes were first detected in 1996 in a *K. pneumoniae* clinical isolate in the USA (Yigit *et al.* 2001), and for many years it was thought to be geographically restrained. Nowadays there are over 15 variants (www.lahey.org/studies/; last accession June 2014) that have been detected worldwide in several Enterobacteriaceae, *Pseudomonas* spp and *Acinetobacter* spp. (Patel and Bonomo 2013).

As other beta-lactamases that have successfully disseminated at a world scale, also KPC carbapenemases owe their dispersion record to their association to diverse genetic platforms with great mobilization potential but also to successful clones (Woodford *et al.* 2011). Moreover, despite the fact that these enzymes are able to virtually hydrolyze all beta-lactams, KPC carbapenemases have never been detected alone, that is, as a single resistance mechanism. Often, KPC enzymes are found together with penicillinases such as TEM-1 and also with ESBLs, most commonly of the SHV-family or with OXA-ESBLs (Patel and Bonomo 2013, Queenan and Bush 2007). Additionally, bla_{KPC} genes have been rarely detected in the chromosome (Patel and Bonomo 2013), but frequently mapped to plasmids that carry supplementary genetic determinants of resistance to other classes of antibiotics as aminoglycosides and quinolones, posing an extra concern for the treatment of infections caused by these KPC-producers (Castanheira *et al.* 2009, Patel and Bonomo 2013).

Finally, the class D carbapenemases comprise a very diverse group of enzymes, mapped both plasmidic- and chromosomally. These OXA carbapenemases have been identified mostly in outbreaks of carbapenems-resistant *Acinetobacter* spp. (e.g. OXA-23, OXA-24, OXA-40, OXA-58), but also in *Pseudomonas* spp. (OXA-50) and Enterobacteriaceae (OXA-48) (Evans and Amyes 2014). Others have been considered as species-specific like the OXA-60 family, naturally present in the genome of *Ralstonia pickettii* (Girlich *et al.*, 2004) and OXA-62 in *Pandoraea pnomenusa* (Schneider *et al.*, 2006).

The emergence of $bla_{\text{OXA-48-like}}$ in Enterobacteriaceae is an example of current antibiotic resistance evolution and dissemination in clinical settings. The carbapenemase OXA-48 was identified for the first time in 2001 in Turkey, in a clinical *K. pneumoniae* isolate (Poirel *et al.* 2004). Initially the dissemination of $bla_{\text{OXA-48}}$ gene was constrained to the Mediterranean region; however, these genes rapidly disseminated to other geographic regions and have now been detected in many European countries, in America, Asia and Australia (Castanheira *et al.* 201, Espedido *et al.* 2013, Mathers *et al.* 2012, Patel and Bonomo 2013, Poirel *et al.* 2012a) (FIG. 6).

OXA-48-like enzymes hydrolyse penicillins and carbapenems, but not extended spectrum cephalosporins. Yet, there are numerous reports on isolates carrying these enzymes that co-produce extended-spectrum-beta-lactamases, and so, in these cases, strains show resistance towards all beta-lactams (Poirel *et al.* 2012a). A recent study performed with OXA-48 carrying Enterobacteriaceae from European and north-Africa countries, showed that 75% of isolates harboured an ESBL-encoding gene (Potron *et al.* 2013b). OXA-48-like-producers were implicated in large death-causing hospital outbreaks in several countries (Cantón *et al.* 2012a, Voulgari *et al.* 2012).

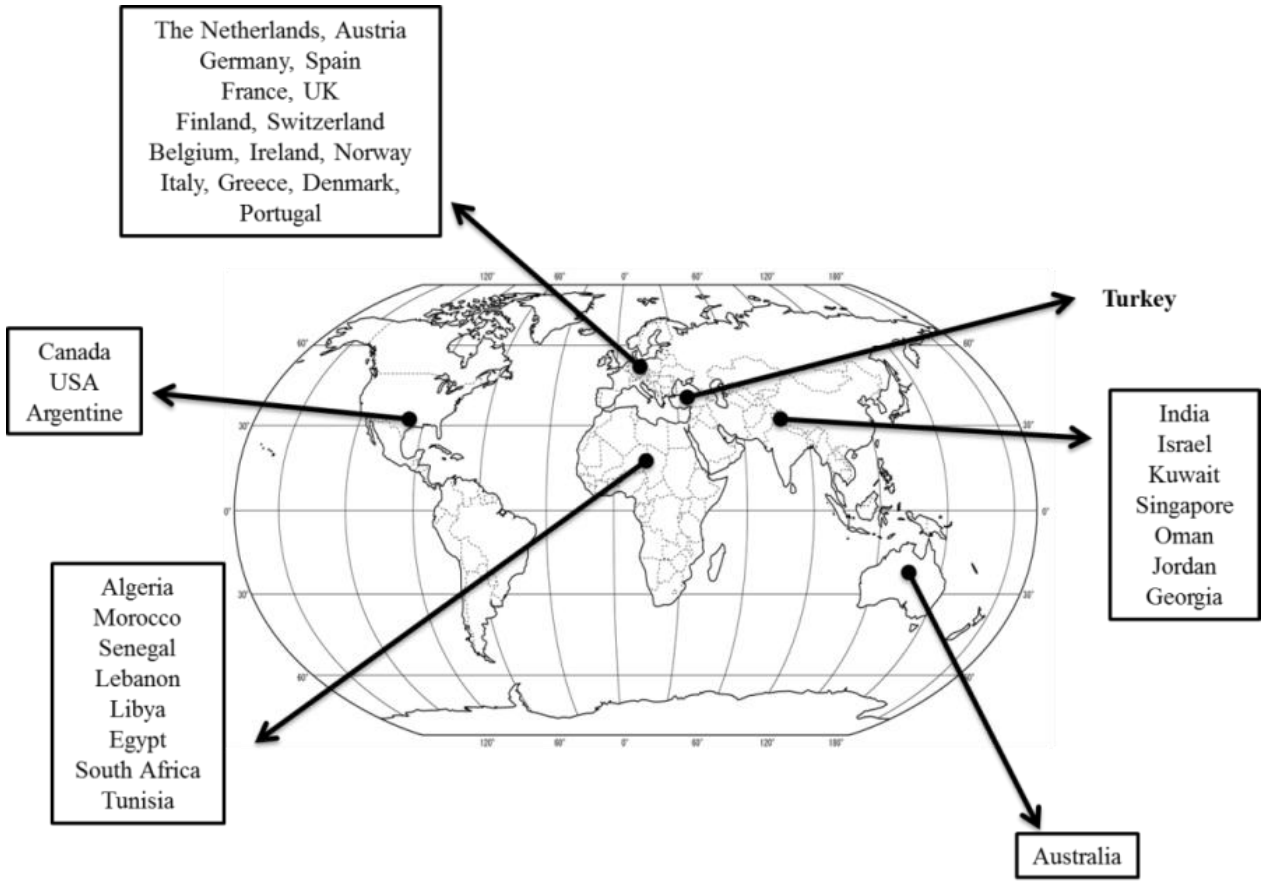

FIG. 6: Worldwide distribution of OXA-48-like carbapenemases.

Several studies underline that OXA-48-like enzymes contribute significantly to carbapenem resistance in Enterobacteriaceae, including some of the most dangerous human pathogens (Castanheira *et al.* 201, Espedido *et al.* 2013, Mathers *et al.* 2012, Patel and Bonomo 2013, Poirel *et al.* 2012a). The water-borne *Shewanella* spp. are the putative origin and reservoir of $bla_{\text{OXA-48-like}}$ (Poirel *et al.* 2004a). There are few reports on $bla_{\text{OXA-48-like}}$ genes outside clinical settings. So far, these genes have been detected in two *Serratia* strains isolated from a river in Morocco (Potron *et al.* 2011), *E. coli* and *K. pneumoniae* from wastewater effluents in Austria (Galler *et al.* 2014), and in Portuguese river waters in *Shewanella xiamenensis* (Tacão *et al.* 2013, chapter 3.2).

So far, 11 sequence variants of OXA-48 like enzymes have been found, with the majority presenting different hydrolytic activity towards carbapenems and differing in 1 to 5 amino acids: OXA-48, OXA-162, OXA-163, OXA-181, OXA-199, OXA-204, OXA-

232, OXA-244, OXA-245, OXA-247 and recently OXA-370 (Poirel et al 2012a, Gomez et al 2013, Sampaio *et al.*, 2014).

While the majority of class D carbapenemases have been recognized as gene cassettes in class 1 integrons, in Enterobacteriaceae the analysis of the genetic context of *bla*$_{\text{OXA-48-like}}$ genes has shown their association to insertion sequences such as IS*Ecp1* and IS*1999*. The fact that different gene variants have been associated to different genetic contexts suggests independent mobilization events, probably from different *Shewanella* species. Also, in Enterobacteriaceae, *bla*$_{\text{OXA-48-like}}$ genes have frequently been found to be plasmid borne. The *bla*$_{\text{OXA-48}}$ gene has been mapped to IncL/M plasmids carrying no additional antibiotic resistance genes (Poirel *et al.* 2012a) and *bla*$_{\text{OXA-204}}$ to IncA/C plasmids (Potron *et al.* 2013). *bla*$_{\text{OXA-181}}$ genes have been associated to IncT plasmids (Villa *et al.* 2013) but also to ColE-type plasmids which are non-conjugative but mobilizable plasmids (Poirel *et al.* 2012a, Sidjabat *et al.* 2013). Also *bla*$_{\text{OXA-232}}$ has been associated to ColE-type plasmids (Potron *et al.* 2013a). Recently it was detected a new variant, in an *E. cloacae* strain isolated in Brazil, designated as OXA-370 (Sampaio *et al.* 2014). The *bla*$_{\text{OXA-370}}$ presented a different genomic context than those reported so far, and it was mapped in an IncF-like plasmid (Sampaio *et al.* 2014).

1.4 THE ENVIRONMENTAL ANTIBIOTIC RESISTOME

The concept of antibiotic resistome has been defined by Wright as the assemblage of all antibiotic resistance genes found in pathogenic or non-pathogenic bacteria and/or antibiotic producers, either free-living in the environment or as commensals of other organisms (Wright 2007). Outside clinical institutions, the detection of antibiotic resistant bacteria and/or antibiotic resistance genes has been reported in a wide range of settings, even in extreme environments or remote locations where no anthropogenic pressure has been exerted (Batt *et al.* 2006, De Souza *et al.* 2006, Miteva *et al.* 2004). Some examples of non-clinical settings where antibiotic resistance has been detected are shown in Table 3.

TABLE 3: Examples of non-clinical settings where antibiotic resistance genes and/or antibiotic resistant bacteria have been detected worldwide.

SETTING	REFERENCES
Hospital sewages and urban wastewaters	Galler *et al.* 2014, Korzeniewska and Harnisz 2013, Moura *et al.* 2012, Novo *et al.* 2013, Ojer-Usoz *et al.* 2014, Rizzo *et al.* 2013
River water and sediment	Aubron *et al.* 2005, Chen *et al.* 2010, Chouchani *et al.* 2013, Girlich *et al.* 2010, Liang *et al.* 2013, Lu *et al.* 2010, Potron *et al.* 2011, Tacão *et al.* 2012, Tacão *et al.* 2013, Tacão *et al.* 2014
Soils	Heuer *et al.* 2011
Estuarine water	Azevedo *et al.* 2013, Henriques *et al.* 2006, Pereira *et al.* 2013
Fountains and wells	Carvalho *et al.* 2012, Henriques *et al.* 2004, Henriques *et al.* 2012
Drinking water	Falcone-Dias *et al.* 2012, Vaz-Moreira *et al.* 2011
Food products	Campos *et al.* 2013, Marti *et al.* 2013, Raphael *et al.* 2011
Farm animals	Fischer *et al.* 2012, Poirel *et al.* 2012b, Poirel *et al.* 2012d, Smet *et al.* 2012, Su *et al.* 2011, Zhu *et al.* 2013
Wild animals	Fischer *et al.* 2013, Poeta *et al.* 2008, Sousa *et al.* 2014, Vredenburg *et al.* 2014
Companion animals	Lloyd 2007, Schmiedel *et al.* 2014, Shaheen *et al.* 2013, Stolle *et al.* 2013

It has been estimated that there are around $5x10^{30}$ bacteria on Earth and the vast majority are inhabitants of soil and water habitats (Whitman *et al.* 1998). As globally environmental bacteria are much more numerous and diverse than human pathogens, there is a growing interest on studying these microorganisms and their habitats. Moreover, increasing evidences on their relevance in much of the resistance mechanisms found in clinical settings has been already emphasized.

A recent study by D'Costa and coworkers has shown that antibiotic resistance mechanisms are ancient and naturally occur in the environment, predating the antibiotic era (D'Costa *et al.* 2011). Most antibiotics used nowadays derived from environmental microorganisms. It is therefore not surprising that neighboring microorganisms and the antibiotic producer itself have developed mechanisms to resist the drugs action (Allen *et al.* 2010, Baquero *et al.* 2009, Davies and Davies 2010, D'Costa *et al.* 2011). These genetic

determinants of resistance could be present in the same gene cluster as the antibiotic biosynthesis pathway gene (Allen *et al.* 2010). In some cases these genes may encode for multifunctional resistance proteins, as for example efflux pumps that allow tolerance to several toxic compounds present in the surrounding environment, including heavy metals and antibiotics (Martinez *et al.* 2009).

For some clinically-relevant resistance mechanisms it has been found an environmental origin. These include for example the widely disseminated $bla_{\text{CTX-M}}$ and $bla_{\text{OXA-48}}$ genes which have their putative origin in environmental *Kluyvera* spp. (Poirel *et al.* 2002) and *Shewanella* spp. (Poirel *et al.* 2004a), respectively, as described above. Also the putative origin of genetic determinants of resistance to quinolones (Qnr-like) has been associated to environmental *Shewanella* and Vibrionaceae members (Poirel *et al.* 2005, Poirel *et al.* 2005a).

Human and veterinary medical institutions are well known hotspots for the acquisition and dissemination of resistance genes and resistant bacteria, due to the high selective pressure resulting from the use of antibiotics. However, the elimination of antibiotics and drug-resistant bacteria or drug resistance genes in subsequent wastes in natural settings as aquatic systems, originates environmental antibiotic resistance hotspots (FIG. 7).

The majority of the antibiotics is soluble in water and can be excreted in urine and faecal matter (Halling-Sørensen 1998, Sarmah *et al.* 2006). Moreover, it has been shown that a significant number of the administered antibiotics may be excreted into the environment still in the active form (Andersson and Hughes 2012, Kümmerer 2009).

Although the concentrations reported for several antibiotics in soil, sediments, surface and ground water are generally low, generally below minimum inhibitory concentrations (Kümmerer 2009), some antibiotics persist in the environment long after their disposal (Heberer 2002, Kay *et al.* 2004, Monteiro and Boxall 2010). Thus, the presence of these not metabolized substances in low concentrations constitutes a selective pressure that, exerted on the resident bacterial population, favors multiplication of resistant strains and promotes processes like horizontal transfer of resistance genes.

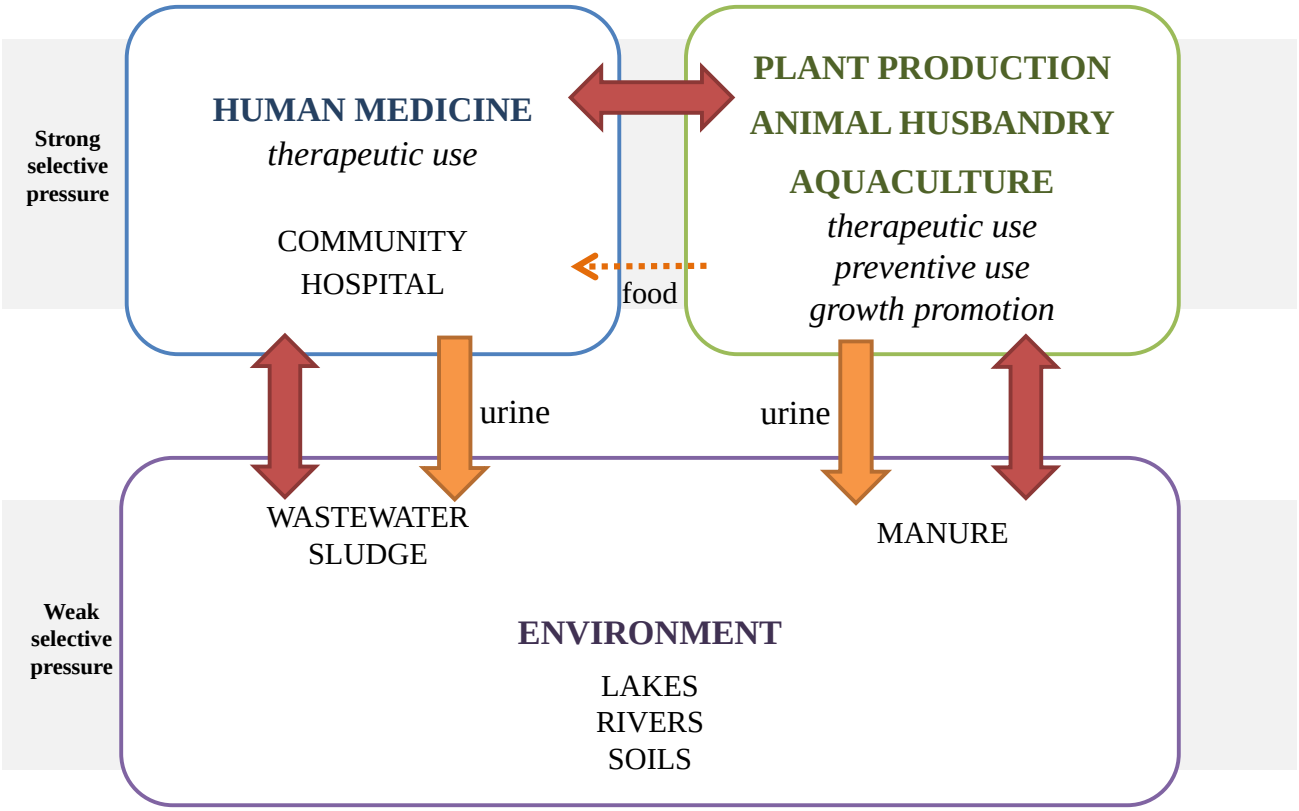

FIG. 7: Schematic representation of flows of resistant bacteria (red) and antibiotics (orange) between settings where antibiotics exert strong (clinical and agricultural) and weak selective pressure (environment) (adapted from Andersson and Hughes 2012).

Antibiotics have been applied not only for the treatment of human and animal infections, but also as food additives in agricultural settings (animal farms, aquacultures, and plantation crops) to promote growth and/or prevent diseases (Cabello *et al.* 2013, Kümmerer 2009, Martinez 2009a, Martinez 2009b, McManus *et al.* 2002). Most antibiotics used as growth promoters are identical to those prescribed in human medicine. In the European Union, the use of antibiotics as food additives in animal farms is banned since 2006 (Regulation no. 1831/2003). Nevertheless these practices went on since the early 1950's, and so contributing to the selection of resistant microorganisms (Jukes and Williams 1953).

A major concern is the fact that subtherapeutic dosage of antibiotics in animal feeds is contributing to the prevalence of antibiotic resistant commensal and pathogenic bacteria. As Alexander Fleming already had observed in 1945 "*…it is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them*" (Fleming 1945). Recent investigations by Gullberg and coworkers have shown that very low concentrations of clinically relevant antibiotics, as present in many natural environments, are relevant for the enrichment and maintenance of resistance as well as for the selection and dissemination of new resistant microorganisms (Gullberg *et al*. 2011).

Although susceptible bacteria are killed or their growth is inhibited by the antibiotic, some few resistant cells succeed to survive and reproduce. Thus, a resistant bacterial population prevails and can transfer resistance determinants to other pathogenic or non-pathogenic bacteria that may be associated to food products, to farm soil often fertilized with wastewater or manure, to farm workers, or irrigation water (Heuer *et al.* 2011, Wellington *et al*. 2013).

Overall, antibiotics and other pollutants (e.g. disinfectants and heavy metals), antibiotic resistant bacteria and antibiotic resistance genes are discharged in the environment from industrial, agricultural or domestic sources, through wastewater treatment plants, hospital sewage or agricultural run-offs. These continuous discharges in the environment, especially in aquatic systems, potentiate the mix of contaminants and bacteria and even accelerate the horizontal transfer of genetic determinants of resistance between indigenous and incoming bacterial populations, thus altering the ecosystem stability (Allen *et al.* 2010, Baquero *et al.* 2009, Marti *et al.* 2014, Taylor *et al.* 2011, Wellington *et al.* 2013, Zhang *et al.* 2009).

Humans are exposed to resistance environmental hotspots through diverse pathways: from the ingestion of food products that have been exposed to contaminated water or soil (for example through consumption of raw vegetables and fruits grown on soils fertilized with contaminated manure or wastewater), to contact with contaminated water or soil through occupational or recreational activities (Wellington *et al.* 2013, Zhang *et al.* 2009).

Aquatic systems such as rivers, streams and lakes are exposed continuously to different anthropogenic impacts of industrial, domestic and agricultural origins. Thus,

these environmental niches constitute important reservoirs of pathogenic and non-pathogenic bacteria, resistant bacteria and resistance genes, antibiotics, metals, disinfectants. This environmental mixture summons ideal conditions for the rapid spread, maintenance and dissemination of antibiotic resistance. Several investigations have been reporting the presence of antibiotic resistant bacteria and antibiotic resistance genes on diverse environmental compartments, including water habitats. These studies have focused mostly in the study of pathogenic organisms present in aquatic compartments or on the analysis of particular spots where an environmental threat has been identified as for example domestic wastewater or hospital sewage discharges (Allen *et al.* 2010, Baquero *et al.* 2008, Wright 2007).

1.4.1 Resistance to last-resort antibiotics in aquatic environments

Over time, the importance of natural aquatic systems as relevant resistance reservoirs has been overlooked, competing with attention given towards the alarming increasing levels of resistance in clinical settings worldwide. Nevertheless, in the last few years, research focused on the environmental resistome has increased as there are growing evidences that pathogenic resistant bacteria and antibiotic resistant genes are not restricted to medical institutions. There are strong indications that the putative origins of relevant resistance mechanism towards last-resort antibiotics reside in environmental isolates (Martinez 2009a, Martinez 2009b, Wright 2007, Zhang *et al.* 2009).

Although there are still few studies available focused in river and lake habitats, the presence of clinically important ESBL genes has been reported in water and river sediment, including the widely disseminated $bla_{\text{CTX-M}}$ genes (Table 4). For example, in a study performed by Zurfluh and colleagues, it was found a high prevalence of ESBL producers among Enterobacteriaceae members isolated from lakes and river water in Sweden, a country that has very strict prescription policies (Zurfluh *et al.* 2013).In Portuguese river water, high prevalence of ESBL producers was detected in polluted environments (Tacão *et al.* 2012), mostly carrying also $bla_{\text{CTX-M-like}}$ genes. Moreover, a high diversity of ESBL producing bacteria and of these ESBL genes was found also in

urban river sediment as described by Lu and coworkers (Lu *et al.* 2010). The most frequently identified $bla_{\text{CTX-M-like}}$ genes in these environments are identical to those found in clinical settings.

As commonly found in clinical settings, also environmental ESBL-producers isolated in aquatic settings are usually multiresistant (Ojer-Usoz *et al.* 2014, Tacão *et al.* 2012, Tacão *et al.* 2013). Furthermore, the majority of ESBL genes is detected in diverse mobilizable genetic structures, carrying additional resistance genes (Chen *et al.* 2010, Tacão *et al.* 2014).

TABLE 4: Extended-spectrum-beta-lactamases reported in water habitats.

Enzyme	Species	Source	Country	Reference
TEM	*Escherichia coli*	river	South Korea	Kim *et al.* 2008
	Escherichia coli	wastewater, river	The Netherlands	Blaak *et al.* 2014
	Escherichia coli, Klebsiella pneumoniae	river/lake	Switzerland	Zurfluh *et al.* 2013
	multiple genera	wastewater	Poland	Korzeniewska *et al.*2013
	multiple genera	hospital sewage	Poland	Korzeniewska *et al.*2013a
SHV	*Aeromonas* spp.	river	France	Girlich *et al.* 2010
	Escherichia coli	wastewater	Spain	Ojer-Usoz *et al.* 2014
	multiple genera	wastewater	Poland	Korzeniewska *et al.*2013
	Escherichia coli, Klebsiella pneumoniae	river/lake	Switzerland	Zurfluh *et al.* 2013
	Escherichia coli	wastewater, river	The Netherlands	Blaak *et al.* 2014
	Escherichia coli	river	Poland	Korzeniewska *et al.*2013
	multiple genera	hospital sewage	Poland	Korzeniewska *et al.*2013a
	Escherichia coli	river	China	Chen *et al.* 2010
CTX-M	*Escherichia coli*	sewage	Austria	Reinthaler *et al.* 2010
	Escherichia coli	river	South Korea	Kim *et al.* 2008
	Escherichia coli	river	UK	Dhanji *et al.* 2011
	Escherichia coli	river	China	Chen *et al.* 2010
	Escherichia coli	wastewater, river	The Netherlands	Blaak *et al.* 2014
	Escherichia coli, Pseudomonas sp.	river	Portugal	Tacão *et al.* 2012
	Escherichia coli	wastewater, river	The Netherlands	Blaak *et al.* 2014
	Escherichia coli, Klebsiella pneumoniae	river/lake	Switzerland	Zurfluh *et al.* 2013
	multiple genera	wastewater	Poland	Korzeniewska *et al.*2013
	Escherichia coli	river	Poland	Korzeniewska *et al.*2013
	Escherichia coli	domestic sewage	Austria	Zarfel *et al.* 2013
	Escherichia coli	wastewater	Spain	Ojer-Usoz *et al.* 2014
	multiple genera	hospital sewage	Poland	Korzeniewska *et al.*2013a
VEB	*Aeromonas* media	lake	Switzerland	Picão *et al.* 2008
	Aeromonas spp.	river	France	Girlich *et al.* 2011
PER	*Aeromonas* spp.	river	France	Girlich *et al.* 2011
	Aeromonas allosaccharophila	river	France	Girlich *et al.* 2010b

Carbapenemase genes have also been reported in diverse water habitats (Table 5). In fact there are some cases of carbapenemase genes that have only been identified in environmental strains. Examples are Sfh-I and SFC-1 in *S. fonticola* (Henriques *et al.* 2004, Saavedra *et al.* 2003) and carbapenemase BIC-1 in *P. fluorescens* (Girlich *et al.* 2010). Also, intrinsic resistance towards carbapenems is well documented in Gram-negatives ubiquitous in aquatic environments such as *Aeromonas* spp. and *S. maltophilia* (Lupo *et al.* 2012, Patel and Bonomo 2013). In *S. maltophilia* resistance results from the expression of bla_{L1}, considered intrinsic to this species (Avison *et al.* 2001) while the majority of members of the genus *Aeromonas* show resistance towards carbapenems due to the expression of chromosomal class B metallo-beta-lactamase genes like bla_{CphA} (Massidaa *et al.* 1991, Walsh *et al.* 2005).

Moreover, for several carbapenems-hydrolyzing beta-lactamases the putative origin has been acknowledged to species that are commonly found in natural settings, as for example the class D carbapenemases OXA-23 in *Acinetobacter radioreducens* (Poirel *et al.* 2008) and OXA-48 in *Shewanella* spp. (Poirel *et al.* 2004, Tacão *et al.* 2013).

Clinically-relevant carbapenemases that are currently causing serious health concerns in clinical settings worldwide have also been identified in different aquatic habitats (Table 5). These include the metallo-beta-lactamases IMP, VIM, NDM, and the serine carbapenemases KPC and OXA-48-like (see Table 5 for references). Particularly in river and lake habitats, carbapenemase genes have been identified mostly in Enterobacteriaceae members and *Pseudomonas* spp..

TABLE 5: Carbapenemases reported in water habitats.

Ambler class	Enzyme	Species	Source	Country	Reference
A	BIC-1	*Pseudomonas fluorescens*	river	France	Girlich *et al.* 2010
	SFC-1	*Serratia fonticola*	water	Portugal	Henriques *et al.* 2004
	KPC	Multiple genera	hospital sewage	Brazil	Chagas *et al.* 2011
		Multiple genera	hospital sewage	Brazil	Picão *et al.* 2013
		Escherichia coli	river	Portugal	Poirel *et al.* 2012
		Klebsiella pneumoniae	wastewater	Austria	Galler *et al.* 2013
		Citrobacter freundii, Enterobacter cloacae	hospital sewage	China	Zhang *et al.* 2012
	GES	*Klebsiella pneumoniae*	wastewater	Portugal	Manageiro *et al.* 2014
	IMI	*Enterobacter asburiae*	rivers	USA	Aubron *et al.* 2005
B	Sfh-I	*Serratia fonticola*	water	Portugal	Saavedra *et al.* 2003
	VIM	*Pseudomonas pseudoalcaligenes, P. aeruginosa*	river, wastewater	Portugal	Quinteira *et al.* 2005, 2006
		Klebsiella pneumoniae, Helicobacter pylori	river	Tunisia	Chouchani *et al.* 2013
		Klebsiella pneumoniae	river/lake	Switzerland	Zurfluh *et al.* 2013
		Pseudomonas spp.	rivers	Portugal	Chapter 3.3
		multiple genera	hospital sewage	Spain	Scotta *et al.* 2011
	IMP	*Pseudomonas fluorescens*	wastewater	Italy	Pellegrini *et al.* 2009
		PCR amplicons	wastewater, effluent	Germany	Szczepanowski *et al.* 2009
		Klebsiella pneumoniae	river	Tunisia	Chouchani *et al.* 2013
	NDM	multiple genera	water	India	Walsh *et al.* 2011
		Klebsiella pneumoniae	river	Vietnam	Isozumi *et al.* 2012
		Acinetobacter baumannii	water, hospital sewage	China	Zhang *et al.* 2013
D	OXA-23	*Acinetobacter baumannii*	river	France	Girlich *et al.* 2010a
	OXA-48	PCR -amplicons	wastewater, effluent	Germany	Szczepanowski *et al.* 2009
		Serratia marcescens	river	Morocco	Potron *et al.* 2011
		Escherichia coli, Klebsiella pneumoniae	wastewater	Austria	Galler *et al.* 2013
		Shewanella xiamenensis	rivers	Portugal	Tacão *et al.* 2014
		PCR -amplicons	rivers, estuary	Portugal	Chapter 3.5

Studies so far have shown that the prevalence of carbapenem-resistant bacteria in aquatic environments from countries with restrictive prescription policies is still low (Henriques *et al.* 2012, Chapter 3.3). Moreover it is mostly related to the presence of intrinsically resistant bacteria.

Broad-range cephalosporins and carbapenems are crucial antibiotics for the treatment of serious infections caused by multiresistant strains, and it is imperative to preserve their purpose. The occurrence and diversity of bacteria resistant to these antibiotics in environmental settings, as of the genes encoding this resistance, has been poorly addressed. However, the studies conducted until now suggest that ESBL genes are becoming frequent in the environment. On the other hand the environmental dissemination of genes encoding for resistance to carbapenems may be at an initial stage. To identify and minimize the human-derived impacts that may promote resistance to these antibiotics it is essential to conduct extensive research on this topic. These studies are also essential to design surveillance programs and measures focused on environmental compartments to limit the occurrence and dissemination of ESBL- and carbapenemase producers.

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2

SCOPE OF THE THESIS

2.1 HYPOTHESIS AND GOALS OF THE THESIS

Dissemination of antibiotic resistance represents a major risk to human health, and is not limited to clinical settings. Although, for a long time, the study of the environmental resistome was undervalued, there have been growing evidences on the role of natural environments in the dissemination of antibiotic resistance.

Aquatic environments, such as rivers or lakes, are reservoirs of indigenous resistant bacteria and resistance genes and at the same time, receive those incoming from different human sources. Environmental and pathogenic bacteria are mixed together, and horizontal gene transfer may occur. Also, the same environmental compartments accumulate the disposals of compounds such as antibiotics, disinfectants or metals. Moreover, aquatic systems are extensively used for leisure activities but also to capture water for human or animal consumption or crops irrigation. Thus water promotes the transfer of microorganisms between different compartments, such as hospitals, farms, and aquacultures, thus facilitating the transmission to humans or other animals.

Nowadays antibiotics and antimicrobial resistance genes are seen as emerging contaminants in the environment vigilance and control. Hence, in the last few years, the environmental resistome and mobilome have received increased attention by the scientific community. The majority of studies reported so far have focused on resistance to widely used antibiotics or extremely disseminated resistances. Further research is needed in order to understand the real extent of the problem, with focus on resistance to critically important antibiotics, such as those used for treatment of serious infections. The study of resistance to antibiotics that are last-resort drugs to treat life-threatening infections caused by Gram-negative bacteria is imperative, since the range of therapeutic options is becoming exceptionally reduced.

In what concerns last-resort antibiotics, it is of major interest to address issues such as, origin, evolution and persistence of antibiotic resistance genes and antibiotic resistant bacteria in the environment. Furthermore, it is quite relevant to understand the role of human activities in the dissemination of antibiotic resistance in environmental settings.

Considering the above, the hypotheses of this thesis are:

- *Rivers are reservoirs and disseminators of antibiotic resistance;*
- *Anthropogenic activities potentiate the dissemination of bacterial resistance to last-resort antibiotics in these environments.*

And the main goal is:

- *to characterize and compare the environmental last-resort antibiotic resistome in polluted and unpolluted rivers.*

Specific goals are:

1) to determine the prevalence and diversity of last-resort antibiotics resistant bacteria in polluted and unpolluted rivers;
2) to characterize clinically-relevant antibiotic resistance mechanisms in these environments;
3) to identify antibiotic resistance dissemination mechanisms in polluted and unpolluted rivers;

2.2 STUDY SITE

The Portuguese hydrographic net is quite vast, and includes aquatic systems that present a wide range of water quality status. For this study we have selected the hydrographic basin of Vouga River, located on the central region of Portugal (FIG. 1). The area covered by this hydrographic basin is around 3645 Km^2, encompassing the totality of 14 municipalities and partially 16. Besides Vouga River, its affluents and sub-tributary streams, this hydrographic basin includes also the multi-estuarine ecosystem Ria de Aveiro. Moreover, this hydrographic area comprises from highly populated urban centers as Viseu and Aveiro (around 52000 and 60000 inhabitants, respectively), to sparsely populated regions (http://www.ine.pt/).

Water from this basin is used for different purposes: occupational such as water capture for human consumption, fishing, aquaculture and agriculture, and recreational as for example the use of several fluvial beaches throughout the basin for leisure activities. There are also important industrial units located in this area, which carry out direct discharges in this aquatic system. Major contributors for the pollution load in this basin are paper pulp factories (located next to Vouga River and also its tributary Caima River), and the industries located at the Estarreja industrial complex that produce diverse chemical products and fertilizers (close to a major tributary of Vouga River, the Antuã River). In fact, the Estarreja industrial complex is the second largest chemical industry complex in Portugal, producing mostly chloride, ammonium sulfate, ammonium nitrate, chloridric and nitric acid, and also synthetic resins. In activity since the 1950's, this industrial complex has produced a large volume of solid and liquid toxic residues that for many years were discharged in the surrounding regions with no regulation enforced (http://www.apambiente.pt/).

For this study twelve rivers from the hydrographic basin of Vouga River were selected. Sampling sites were chosen taken into account information available from the entities responsible for managing hydrographic basins in central Portugal (http://www.apambiente.pt/). This data include for example the location of industries, animal farms and aquacultures in this region (FIG. 1).

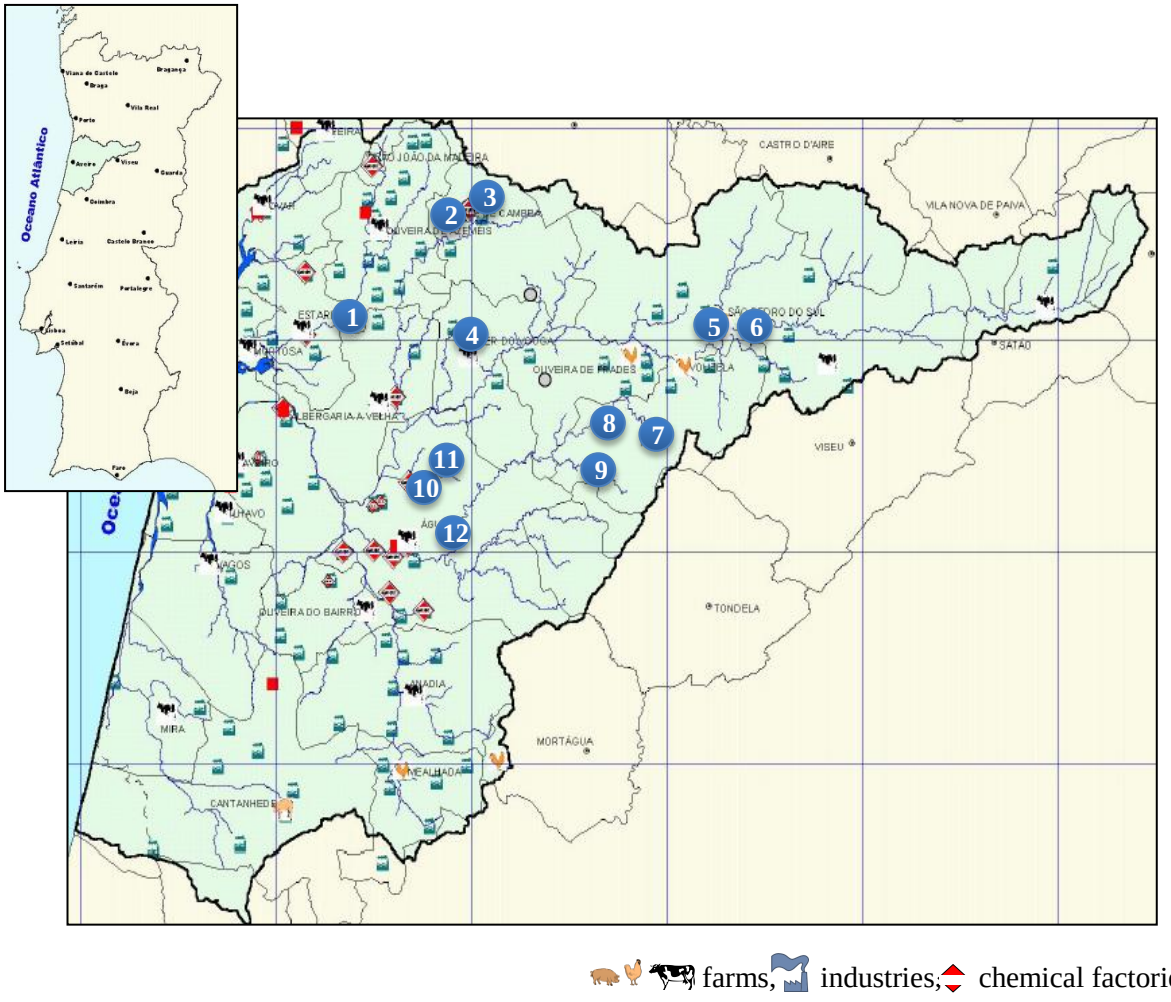

FIG. 1: Map of Vouga River basin (Central Portugal) with the location of the 12 sampling sites included in this study (1- River Antuã, 2- River Úl, 3- River Ínsua, 4- River Caima, 5- River Zela, 6- River Vouga, 7- River Alcofra, 8- River Alfusqueiro, 9- River Águeda, 10- River Águeda, 11- River Da Póvoa, 12- River Cértima). Labels indicate industrial and agricultural activities in the region (available at http://www.apambiente.pt/).

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3

RESULTS AND DISCUSSION

Part I

RESISTANCE TO 3RD GENERATION CEPHALOSPORINS IN NATURAL ENVIRONMENTS

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3.1

RESISTANCE TO BROAD-SPECTRUM ANTIBIOTICS IN AQUATIC SYSTEMS: ANTHROPOGENIC ACTIVITIES MODULATE THE DISSEMINATION OF *bla*$_{\text{CTX-M-LIKE}}$ GENES

Abstract

We compared the resistome within polluted and unpolluted rivers, focusing on extended-spectrum beta-lactamases (ESBL) genes, in particular *bla*$_{\text{CTX-M}}$. Twelve rivers from a Portuguese hydrographic basin were sampled. Physicochemical and microbiological parameters of water quality were determined and results classified 9 rivers as unpolluted (UP) and 3 as polluted (P). Of the 225 cefotaxime-resistant strains isolated, 39 were identified as ESBL producers, with 18 carrying a *bla*$_{\text{CTX-M}}$ gene (15 from P and 3 from UP). Analysis of CTX-M nucleotide sequences showed that 17 isolates produced CTX-M from group 1 (CTX-M-1, -3, -15 and -32) and 1 gene belonged to group 9 (CTX-M-14). The genetic environment study revealed the presence of different genetic elements previously described in clinical strains. IS*Ecp1* was found in the upstream region of all isolates examined. Culture-independent *bla*$_{\text{CTX-M}}$-like libraries comprised 16 CTX-M gene variants, 14 types in the P library and 4 types in UP library, varying from 68% to 99% similarity between them. Besides the much lower diversity among UP CTX-M-like genes, the majority were similar to chromosomal ESBLs such as *bla*RAHN-1. The results demonstrate that occurrence and diversity of *bla*$_{\text{CTX-M}}$ genes are clearly different between polluted and unpolluted lotic ecosystems; these findings favor the hypothesis that natural environments are reservoirs of resistant bacteria and resistance genes, where anthropogenic-driven selective pressures may be contributing to the persistence and dissemination of genes usually relevant in clinical environments.

3.1.1 INTRODUCTION

Antibiotics are widely used not only to treat human and animal infections but also in farms and aquacultures as food additives to promote animal growth and prevent diseases. Consequently, antibiotics are released in large amounts in natural ecosystems where they can impact the structure and activity of environmental microbial populations (Martinez 2009, 2009a).

Undoubtedly, the occurrence and dissemination of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are recognized worldwide as a major public health concern. Efforts on prevention of ARGs and ARB spread focused on a clinical and human-community level, being especially centered on infection control and restriction of antibiotic use (Taylor *et al.* 2011). However, considering the growing evidences that ARGs and pathogenic ARB are no longer restricted to clinical settings, it is quite clear that the research activities need to be expanded to include non-pathogenic environmental microorganisms that could be the potential source for these ARGs (Martinez 2009, Martinez 2009a, Wright 2010, Zhang *et al.* 2009).

Aquatic systems can be highly impacted by human activities receiving contaminants and bacteria from different sources and thus encouraging the promiscuous exchange and mixture of genes and genetic platforms. Consequently these systems may promote the spread of ARB and ARGs and even the emergence of novel resistance mechanisms and pathogens (Ash *et al.* 2002, Baquero *et al.* 2008, Zhang *et al.* 2009). Considering the frequent detection of ARGs and ARB in aquatic systems and since their dissemination constitutes a serious public health problem, it has been suggested that ARGs should be considered as environmental emerging contaminants (Martiz 2009a, Pruden *et al.* 2006).

Beta-lactam antibiotics are the most broadly used antibacterial agents. Extended-spectrum beta-lactamases (ESBLs) mediate resistance to broad-spectrum beta-lactams such as cefotaxime and ceftazidime, and are widely disseminated among Gram-negative bacteria. Since first reported in 1983 (Kliebe *et al.* 1985), the occurrence of infections caused by ESBL-producing bacteria has been constantly rising and constitutes a serious threat to human health. CTX-M genes have rapidly become the most common ESBL genes

mainly because of the genetic platforms responsible for their mobilization and dissemination (insertion sequences, integrons, transposons, plasmids). Particularly common on the genomic environment of these genes are insertion sequences such as IS*Ecp1*, IS*26* and IS*CR1* (Bush and Fisher 2011, Cantón and Coque 2006, Coque et al. 2008). CTX-M-15 and CTX-M-14 are the most prevalent enzymes, over 110 CTX-M-like ESBLs described so far, mostly found in Enterobacteriaceae but also, for example, in *Aeromonas* spp., *Pseudomonas* spp. and *Acinetobacter* spp. (Chen *et al.* 2010, Coque *et al.* 2008, Novais *et al.* 2010, Woodford *et al.* 2011). Interestingly, the CTX-M-like ESBLs are thought to have evolved from chromosomal genes of the non-clinical genus *Kluyvera* (Poirel *et al.* 2002). Few studies addressed the links between pollution and the dispersal of ARB and ARGs in natural environments. It is of major importance to understand how anthropogenic activities are modulating the resistance gene pool in order to anticipate future impacts and consequences for the environment and public health. Also, ARGs, and specifically those most frequently found in association with pathogenic bacteria such as CTX-M genes, may be key indicators of water quality and may be used to trace the dissemination of multiresistance in aquatic environments.

In this study our goal was to compare the cefotaxime resistome within polluted and unpolluted lotic (flowing waters) ecosystems. Specific goals were: 1) to compare the occurrence and phylogenetic diversity of cefotaxime-resistant bacteria and ESBL producers; 2) to detect and characterize the ESBL genes responsible for the resistance phenotype; 3) to compare the diversity of CTX-M-like genes using culture-dependent and culture-independent approaches.

3.1.2 MATERIAL AND METHODS

3.1.2.1 Samples collection and water quality assessment

Water samples were collected in 12 sites from 11 rivers integrated in the Vouga River basin, located in central Portugal (FIG. 1). Table S1 in the supplemental material indicates

the Global Positioning System (GPS) coordinates of all sampling locations. Throughout the basin, these water bodies are exposed to different anthropogenic impacts from agricultural, industrial and domestic origins, which results in different levels of superficial water quality from unpolluted to extremely polluted sites (DRA 1998). Sampling sites were selected in order to include from putative unpolluted to extremely polluted sites.

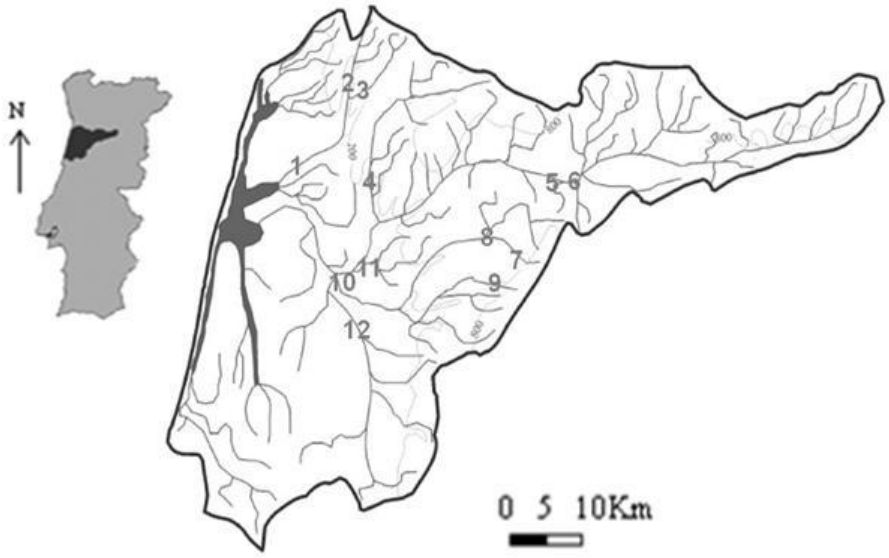

FIG. 1: Map of Vouga River basin (Central Portugal) with the location of the 12 sampling sites under study.

Water was collected in sterile bottles (7L) from 50 cm below the water surface and kept on ice for transportation. To infer as to the water quality, physical, chemical and microbiological parameters were determined according to Portuguese laws (Government of Portugal 1998), which included pH, color, smell, dissolved oxygen, conductivity, temperature, nitrates, chlorides, phosphates, ammonium, chemical oxygen demand, biological oxygen demand, total and fecal coliforms and fecal streptococci. Surface water quality classification was assigned according to regulations given by the national institute of water (www.inag.pt) which sorts water quality in 5 categories from unpolluted to extremely polluted water in accordance with parameters established by the Portuguese law.

3.1.2.2 Enumeration and selection of cefotaxime resistant bacteria

Water samples were filtered in 0.45-µm-pore-size cellulose ester filters (Pall Life Sciences, MI, USA), and the membranes placed on MacConkey agar plates supplemented with 8 µg/ml of cefotaxime to select cefotaxime resistant isolates. Also, to determine the proportion of cefotaxime resistant bacteria among the total bacterial population, plates with no antibiotic supplement were used. Plates were then incubated at 37ºC for 16 h. Colony counting was done in triplicate. Individual cefotaxime-resistant colonies were purified and stored in 20% glycerol at −80ºC.

3.1.2.3 Molecular typing and identification of cefotaxime resistant isolates

Genomic DNA was isolated as previously described (Henriques *et al.* 2004). BOX-PCR was used to type all isolates as previously described (Tacão *et al.* 2005). PCR products were loaded in 1.5% agarose gels for electrophoresis. The banding patterns were analyzed with the software GelCompar (Applied Maths, Belgium). Similarity matrices were calculated with the Dice coefficient. Cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages (UPGMA).Isolates displaying different BOX profiles were identified by 16S rRNA gene sequencing analysis with primers and PCR conditions as previously described (Henriques *et al.* 2004). PCR products were purified with the JETQUICK PCR purification spin kit (GENOMED, Löhne, Germany) and used as template in the sequencing reactions. Online similarity searches were performed with the BLAST software at the National Center of Biotechnology Information website.

3.1.2.4 Antibiotic susceptibility testing and ESBL detection

Antimicrobial resistance patterns were determined by the agar disc diffusion method on Mueller–Hinton agar, against 16 antibiotics from 6 classes: beta-lactams (penicillins,

monobactams, carbapenems and 1st, 3rd and 4th generation cephalosporins), quinolones, aminoglycosides, phenicols, tetracyclines and the combination sulfamethoxazole/trimethoprim. Discs containing the following antibacterial agents were used: amoxicillin (10 μg), amoxicillin/clavulanic acid (20 μg/10 μg), ampicillin (10 μg), aztreonam (30 μg), cefepime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cephalothin (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), gentamicin (10 μg), imipenem (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), sulfamethoxazole/trimethoprim (25 μg) and tetracycline (30 μg) (Oxoid, Basingstoke, UK). After 24 h of incubation at 37ºC, organisms were classified as sensitive, intermediate, or resistant according to the Clinical Laboratory Standards Institute guidelines (7). Detection of ESBL production was carried out by the double-disc synergy test (DDST) (18) and a clavulanic acid combination disc method, based on comparing the inhibition zones of cefpodoxime (10 μg) and cefpodoxime-plus-clavulanate (10/1 μg) discs (Oxoid, UK). Statistical analysis was performed by two-sample t-test with a critical P-value set at 0.05.

3.1.2.5 ESBLs and integrase genes screening

PCR screening was performed for ESBLs genes encoding SHV, TEM, OXA, CTX-M (group 1, 2, 8/25 and 9), GES, VEB and PER, with primer sets and PCR conditions as described elsewhere (Dallenne *et al.* 2010, Henriques *et al.* 2006). Integrase screening was performed for *intI*1, *intI*2 and *intI*3 genes (Dallenne *et al.* 2010, Henriques *et al.* 2006, Moura *et al.* 2012). Genomic DNA of positive control strains was used (16, 24). Each experiment included as negative control a PCR reaction containing water instead of DNA. Amplicons were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

3.1.2.6 Diversity and genetic environment of bla_{CTX-M} genes

Sequencing was done for the *bla*$_{CTX-M}$ gene fragments amplified from the bacterial isolates. The presence of IS*Ecp1*, IS*26*, IS5, orf*477*, IS*903* and orf*503* in the genetic environment of *bla*$_{CTX-M}$ was searched by PCR (Eckert *et al.* 2006, Fernandez *et al.* 2007, Saladin *et al.* 2002).

3.1.2.7 Construction of bla_{CTX-M} gene libraries

To investigate further the diversity of the *bla*$_{CTX-M}$ genes in both polluted and unpolluted environments environmental DNA from water samples was isolated as previously described (Henriques *et al.* 2004). DNA isolated from all polluted sites was mixed, as also from unpolluted samples. Hence, two clone libraries of *bla*$_{CTX-M}$ were constructed using the TA Cloning Kit, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The *bla*$_{CTX-M}$ gene was amplified using the CTX-F and CTX-R primer set (Lu *et al.* 2010). Clones were screened by PCR for the presence of fragments with the expected size by using primers targeting the vector. PCR products were purified and sequenced. Similarity searches were performed using BLAST. A phylogenetic tree was obtained using MEGA version 5 (Tamura *et al.* 2011). The Shannon–Weaver index of diversity (H) was calculated for each library using the formula $H = - \Sigma(n_i/N) \log(n_i/N)$, where ni is the abundance of each *bla*$_{CTX}$ type and N is the sum of analyzed clones in each library.

3.1.2.8 Nucleotide sequence accession numbers

All *bla*$_{CTX-M}$ genes nucleotide sequences reported in this work have been deposited in the GenBank database under the accession numbers JQ397652–JQ397669 (bacterial strains) and JQ397670–JQ397721 (clone libraries). Also 16S rRNA gene sequences are available with the accession numbers JQ781502-JQ781652.

3.1.3 RESULTS

3.1.3.1 Water quality and occurrence of cefotaxime-resistant bacteria

From the analysis of all physical, chemical and microbiological parameters (see Table S2 in the supplemental material) and according to Portuguese law (D.L. 236/98) and the surface water quality classification given by the national water institute, from the 12 sites under study, 3 sites were classified as polluted (P) and 9 as unpolluted (UP). All three rivers classified as polluted presented a mixed type of pollution, mainly related to exceptionally high values of phosphates and total coliforms (Table S2 in the supplemental material; D.L. 236/98).

The total bacterial counts on MacConkey agar in polluted sites was on average 1.9 X 10^5 CFU/100mL of riverine water of which 8.8% grew on MacConkey agar supplemented with cefotaxime (1.7X10^4 CFU/100mL), and in pristine rivers was on average 0.68 X 10^5 CFU/100mL of which 0.6% grew on MacConkey agar supplemented with cefotaxime (4.4 X10^2 CFU/100mL).

3.1.3.2 Molecular typing and identification of bacterial isolates

Clonal relationships among cefotaxime resistant isolates (n=225) were assessed by BOX-PCR, and 151 isolates displaying unique BOX profiles were selected for further analysis (see FIG. S1 in the supplemental material). Among strains isolated from polluted waters (n= 60), 41.7% were identified as *Pseudomonas* spp. (*P. fluorescens, P. nitroreducens, P. plecoglossicida and P. putida*), 35% affiliated with Enterobacteriaceae members and 21.7% with *Aeromonas* spp.. The Enterobacteriaceae members mostly affiliated with *Escherichia coli* (25%), followed by *Enterobacter* spp. (8.33%) and with only an isolate each *Alcaligens faecalis* and *Citrobacter freundii*.

As of unpolluted waters isolates (n=91) *Pseudomonas* spp. (*P. fluorescens, P. nitroreducens and P. putida*) adds 63.7%, Enterobacteriaceae and *Aeromonas* spp. (*A. media* and *A. hydrophila*) with 8.8% and 1.1% respectively, and *Acinetobacter* sp. appears as the second most abundant genus in these samples, with 26.4% (all *Acinetobacter calcoaceticus*). Among Enterobacteriaceae members, *Enterobacter* sp. and *E. coli* were identified (5.5% and 3.3%, respectively). A 16S rRNA gene phylogenetic tree is presented in supplemental material FIG S2.

3.1.3.3 Antimicrobial susceptibility and detection of ESBL producers

As expected, since isolates were selected in agar plates supplemented with cefotaxime, higher numbers of antibiotic resistance were registered for beta-lactams (see FIG. S3 in the supplemental material). It was determined that 22.5 % of the isolates from P and UP samples were resistant to all cephalosporins tested and 52.3% resistant to both cefotaxime and ceftazidime. For beta-lactams, higher percentages (although not statistically significant; two-sample *t* test, $P > 0.05$) were always observed for isolates from polluted waters. For non-beta-lactam antibiotics higher resistance levels were observed against quinolones (in particular nalidixic acid with 78.1% resistants), sulfamethoxazole-trimethoprim and chloramphenicol (55% and 51%, respectively). In isolates from polluted environments also resistance to tetracycline (36.7%) and to aminoglycosides (31.7%) was frequently detected. Besides imipenem (99.3% susceptible strains), gentamicin was the most effective, with only 3.3% resistance among isolates from UP and 21.7% from P sites. The less effective were the penicillins, the monobactam aztreonam and 1st and 3rd generation cephalosporins. Significant differences were found among isolates from polluted and unpolluted waters in resistance frequencies towards aminoglycosides, quinolones, tetracycline and the combination sulfamethoxazole/trimethoprim (two-sample t test, $P < 0.05$).

Multiresistance (defined as resistance to 3 or more classes of antibiotics, including beta-lactams) was found in 56.6% and 46.0% of the strains isolated from polluted and unpolluted sites, respectively.

Of the 151 isolates tested, 39 were positive for ESBL production by both used methods, with 27 isolates from polluted waters (13 *E. coli*, 8 *Aeromonas* spp. and 6 *Pseudomonas* spp.) and 12 isolates from unpolluted sites (7 *Pseudomonas* spp., 2 *Acinetobacter* sp., 2 *E. coli* and 1 *Aeromonas* spp.).

3.1.3.4 Occurrence and diversity of integrases and ESBLs genes

The ESBL-producing isolates were further analyzed by PCR screening for ESBLs and integrase genes. As for ESBL genes the most frequently detected was $bla_{\text{CTX-M}}$ (n=18) followed by bla_{TEM} (n=10). In 6 strains it were identified both $bla_{\text{CTX-M}}$ and bla_{TEM}. Two bla_{VEB} were identified, both on *Aeromonas* sp., once in each environment. OXA-1-like genes were detected in 6 strains isolated from polluted sites. No bla_{GES}, bla_{PER}, bla_{SHV} or $bla_{\text{OXA-2}}$ and $bla_{\text{OXA -10-like}}$ were identified with the primer sets used in this study.

Integrase genes *intI1*, *intI2* and *intI3* were screened by PCR among the 39 ESBL-producers. On 22 out of 39 isolates it was detected *intI1* (19 P and 3 UP), affiliated with *Escherichia coli* (11 P and 1 UP), *Pseudomonas* sp. (2 P and 1 UP) and *Aeromonas* sp. (6P and 1UP). The *intI2* and *intI3* genes were not detected.

3.1.3.5 Diversity and genetic environment of $bla_{\text{CTX-M}}$ genes

Since $bla_{\text{CTX-M}}$ was the most frequently detected among the 39 ESBL-producers, $bla_{\text{CTX-M}}$ genes were further characterized (Table 1).

TABLE 1: Characteristics of the *bla*$_{CTX-M}$ producers isolated from polluted (P) and unpolluted (UP) samples, regarding phylogenetic affiliation, sample origin, ESBL and integrase genes detected and antimicrobial resistance profile.

Isolate	Phylogenetic affiliation	Sample (P/UP)	ESBL genes detected by PCR	Antibiotic resistance profile	*IntI 1*
E1	*A. hidrophila.*	P	*bla*$_{TEM}$, *bla*$_{CTX-M}$	AML, AMP, AMC, KF, CTX, FEP, CIP, NA, CN, K, TE	+
E2	*A. hydrophila*	P	*bla*$_{TEM}$, *bla*$_{CTX-M}$	AML, AMP, AMC, KF, CTX, FEP, NA, CN, K, TE	+
E3	*A. hydrophila*	P	*bla*$_{TEM}$, *bla*$_{CTX-M}$ -M	AML, AMP, AMC, ATM, KF, CTX, FEP, CIP, NA, K, TE	+
E4	*E. coli*	P	*bla*$_{CTX-M}$	AML, AMP, AMC, ATM, KF, CTX, CAZ, FEP, CIP, NA, C, CN, K, TE	-
E5	*E. coli*	P	*bla*$_{CTX-M}$, *bla*$_{OXA}$	AML, AMP, AMC, ATM, KF, CTX, CAZ, FEP, CIP, NA, CN, K, SXT, TE	+
E6	*E. coli*	P	*bla*$_{TEM}$, *bla*$_{CTX-M}$	AML, AMP, AMC, ATM, KF, CTX, NA, C, TE	+
E7	*E. coli*	P	*bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{OXA}$	AML, AMP, AMC, ATM, KF, CTX, CAZ, FEP, CIP, NA, CN, K, SXT, TE	+
E8	*E. coli*	P	*bla*$_{CTX-M}$, *bla*$_{OXA}$	AML, AMP, AMC, ATM, KF, CTX, CAZ, FEP, CIP, NA, CN, K, SXT, TE	+
E9	*E. coli*	P	*bla*$_{CTX-M}$	AML, AMP, ATM, KF, CTX, CAZ, FEP, CIP, NA, CN, K, SXT, TE	+
E10	*E. coli*	P	*bla*$_{CTX-M}$	AML, AMP, ATM, KF, CTX, CAZ, FEP, CIP, NA, K, SXT, TE	+
E11	*E. coli*	P	*bla*$_{CTX-M}$	AML, AMP, ATM, KF, CTX, CAZ, FEP, CIP, NA, K, SXT, TE	+
E12	*E. coli*	P	*bla*$_{CTX-M}$, *bla*$_{OXA}$	AML, AMP, AMC, ATM, KF, CTX, CAZ, FEP, CIP, NA, CN, K, SXT, TE	+
E13	*E. coli*	P	*bla*$_{TEM}$, *bla*$_{CTX-M}$	AML, AMP, AMC, ATM, KF, CTX, CAZ, FEP, SXT	+
E14	*E. coli*	P	*bla*$_{CTX-M}$	AML, AMP, ATM, KF, CTX, FEP, SXT, TE	+
E15	*E. coli*	P	*bla*$_{CTX-M}$	AML, AMP, AMC, ATM, KF, CTX, CAZ, FEP, NA, SXT, TE	+
E16	*E. coli*	UP	*bla*$_{CTX-M}$	AML, AMP, ATM, KF, CTX, CAZ, FEP, CIP, NA, SXT	+
E17	*E. coli*	UP	*bla*$_{TEM}$, *bla*$_{CTX-M}$	AML, AMP, AMC, ATM, KF, CTX, CAZ, TE	-
E18	*Pseudomonas* sp.	UP	*bla*$_{CTX-M}$	AML, AMP, AMC, ATM, KF, CTX, NA, C, SXT	+

The CTX-M genes were detected in 18 isolates (15P and 3 UP). The nucleotide sequence of *bla*$_{CTX-M}$ genes was determined and their genomic environment was inspected by PCR and sequencing. Sequence analysis showed that isolates produced CTX-M from group 1 (CTX-M-1, -3, -15 and -32) and group 9 (CTX-M-14). The CTX-M-1 gene was found in 3 isolates (all from polluted water), CTX-M-3 gene in 3 isolates (all from polluted water), CTX-M-15 in 10 isolates (8P and 2UP) and CTX-M-32 was detected in only 1 isolate from unpolluted water. From group 9 it was found CTX-M-14 gene in one strain isolated from polluted water. The genetic environment study revealed the presence of 6 different genetic environments with elements previously described in clinical strains. A

schematic representation of the different genomic environments found in the 18 isolates is presented in figure 2.

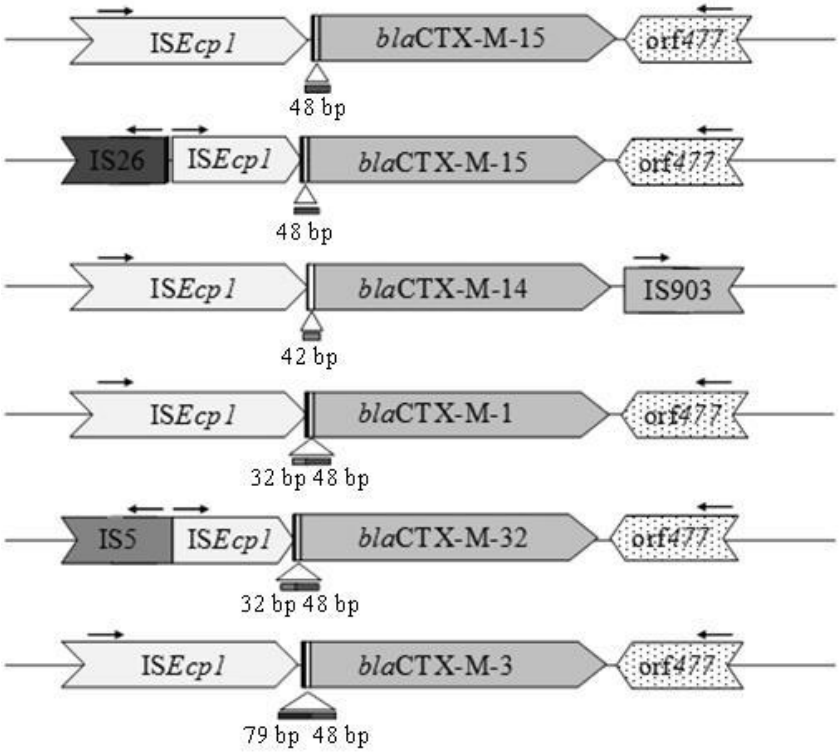

FIG. 2: Schematic representation of the genetic environment of CTX-M genes from the 18 isolates producing CTX-M from group 1 (CTX-M-1, -3, -15 and -32) and group 9 (CTX-M-14). The number of isolates from each polluted and unpolluted environment that carry each variant is indicated.

IS*Ecp1* was found in the upstream region of all isolates examined in the present study, but disrupted in 8 isolates by IS*26* and in 1 by IS*5*. The distance between IS*Ecp1* and the start codon of $bla_{\text{CTX-M}}$ genes was as previously described, varying from 32bp to 127bp. All $bla_{\text{CTX-M}}$ from group 1 presented downstream an Orf477. The only $bla_{\text{CTX-M}}$ from cluster 9 detected was *bla*CTX-M-14 (E6) which presented downstream an IS*903*-like element.

3.1.3.6 Polluted and unpolluted $bla_{CTX\text{-}M\text{-}like}$ clone libraries

To compare the diversity of *bla*$_{\text{CTX-M}}$ genes in polluted and unpolluted environments, two clone libraries of *bla*$_{\text{CTX-M-like}}$ gene fragments were constructed and analyzed. Gene fragments were amplified using as template two environmental DNA pools corresponding each to P and UP samples. A total of 52 clones were obtained and all inserts were sequenced (27 P and 25 UP). Culture-independent *bla*$_{\text{CTX-M-like}}$ libraries comprised 16 gene variants (A-P), 14 types in the P library (H= 1.04) and 4 types in UP library (H= 0.23), with similarity values varying from 68% to 99% between them and from 97% to 100% with sequences from GenBank database. The majority (n=16) affiliated with nucleotide sequences of *bla*$_{\text{CTX-M}}$ variants from group 1 (CTX-M-1, -12. -15, -30, -37, -68 and -97) but also *bla*$_{\text{CTX-M}}$ from group 2 (CTX-M-97) (n=2), group 9 (CTX-M-14) (n=3) and group 25 (CTX-M-78 and -100) (n=2) were identified.

Besides the much lower diversity among UP CTX-M-like genes, the majority were similar to chromosomal ESBLs such as *bla*$_{\text{RAHN-1}}$, *bla*$_{\text{RAHN-2}}$ and *bla*$_{\text{FONA-5}}$ (FIG. 3).

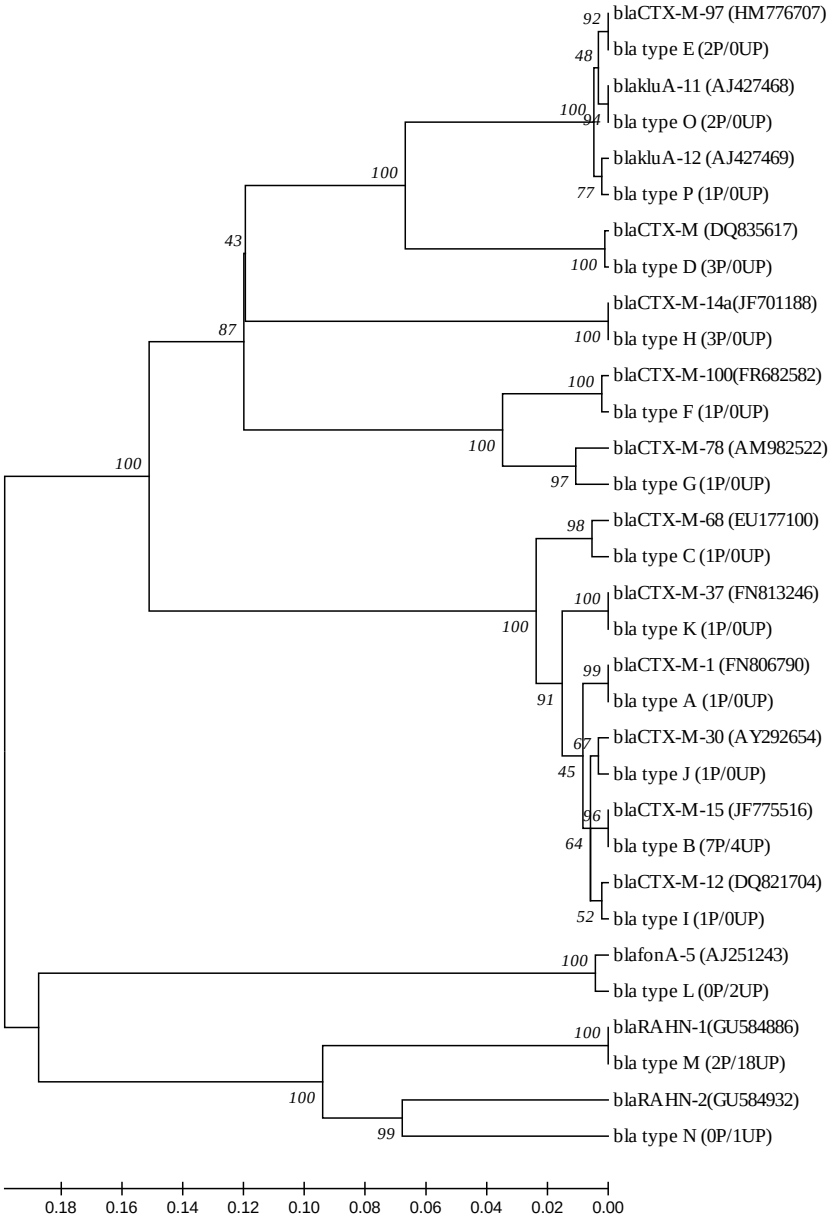

FIG. 3: Dendrogram tree of *bla*$_{\text{CTX-M}}$ gene sequences types A to N identified from the polluted (P) and unpolluted (UP) genomic libraries. The number in parentheses shows the number of times the sequence was found in the library. The branch numbers refer to the percent confidence as estimated by a bootstrap analysis with 1000 replications.

3.1.4 DISCUSSION

Lotic ecosystems are threatened daily by anthropogenic actions that compromise water quality and, in consequence, its sustainable use.

Considering aquatic systems as reactors for diverse biological interactions that have important genetic implications, the study of the aquatic antibiotic resistome (which includes ARGs, pathogenic and non-pathogenic ARBs) is important, as it might indicate the extent of alteration of water ecosystems by anthropogenic activities. Several studies have been reporting the presence of antibiotic resistant bacteria from several aquatic environments but focusing on pathogenic organisms or directly related to an environmental threat as a hospital sewage discharge (Allen *et al.* 2010, Baquero *et al.* 2008, Wright 2010).

In this study, two groups of rivers (polluted and unpolluted), which are part of the same Portuguese lotic ecosystem, were inspected for the presence of cefotaxime-resistant Gram-negative bacteria, in order to understand how human action is modulating the environmental resistome, in particular the cefotaxime-resistome.

As expected, high levels of resistance were obtained in this study, among CTX^R isolates, against other beta-lactams frequently conferred by the same resistance mechanism (16), with higher occurrence among P strains. ESBL-production was detected in *Pseudomonas* sp., *Acinetobacter* sp., *E. coli* and *Aeromonas* sp., and was more frequent among isolates from polluted sites. Recently in several environmental studies, members of the same genera have been identified as ESBL-producers, enforcing their relevance and importance for resistance monitoring (Girlich *et al.* 2011, Guenther *et al.* 2011, Poeta *et al.* 2008). We investigated the presence of different ESBL genes and found $bla_{\text{CTX-M}}$ gene as the most prevalent followed by bla_{TEM} genes. The majority of the isolated CTX-M-producers affiliated with *E. coli* but also with *Aeromonas hydrophila* (3 $bla_{\text{CTX-M-3}}$) and *Pseudomonas* sp. (1 $bla_{\text{CTX-M-15}}$). Few studies have reported the presence of $bla_{\text{CTX-M}}$ genes in *Pseudomonas* spp. and *Aeromonas* spp.. A previous study reported $bla_{\text{CTX-M-27}}$ genes in 2 *Aeromonas* sp. isolated in river sediment (Lu *et al.* 2010).

Also *Aeromonas* spp. producing $bla_{\text{CTX-M-3}}$ and $bla_{\text{CTX-M-15}}$ have been detected in clinical settings and directly implicated in human infections (Ye *et al.* 2010). As far as we know, this is the first work reporting environmental *Aeromonas* spp. producing $bla_{\text{CTX-M-3}}$ genes. Also in *Pseudomonas* spp. reports on CTX-M producers are rare. In fact the majority refer to clinical *Pseudomonas aeruginosa* isolates which have been reported to produce CTX-M-1, -2, -15 and –43 (Picão *et al.* 2009). Also recently 2 spinach saprophyte strains identified as *P. putida* and 1 *P. teessidea* were referred as CTX-M-15-producers (Raphael *et al.* 2011).

To detect any potential genetic platforms able to mobilize the $bla_{\text{CTX-M}}$ genes, we also analyzed the genomic environment of the 18 $bla_{\text{CTX-M}}$ genes detected. Different insertion sequence elements were found. Upstream the *bla* gene in all strains it was detected an IS*Ecp1* element. Other IS elements (IS*5* and IS*26*) were found but disrupting the IS*Ecp1* element. The organization IS*26* and end of IS*Ecp1* has been mostly found in clinical Enterobacteriaceae isolates but it was also described in an *E. coli* $bla_{\text{CTX-M-1}}$ producer isolated from seagulls fecal droppings (Eckert *et al.* 2006, Poeta *et al.* 2008, Saladin *et al.* 2002). On the other hand, the organization IS*5* and end of IS*Ecp1* was found upstream the *bla*CTX-M-32 gene in environmental and clinical *E. coli* isolates (Fernandez *et al.* 2007, Poeta *et al.* 2008). The presence of IS*Ecp1* element upstream $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ has been also reported in clinical isolates (Eckert *et al.* 2006, Lartigue *et al.* 2004, Saladin *et al.* 2002). Downstream of the *bla* genes in the CTX-M-1 group, sequence ORF477 was present in all strains. Another insertion sequence, IS*903*, was found downstream the $bla_{\text{CTX-M-14}}$ from CTX-M group 9, as already described by other authors in clinical Enterobacteriaceae isolates (Eckert *et al.* 2006, Lartigue *et al.* 2004, Saladin *et al.* 2002). The common phenotype of multiresistance among ESBL-producing isolates is a result of the presence of other genes, normally encoded in the same plasmid carrying ESBL genes. This gene panoply contributes to maintaining ESBL-producing bacterial communities, even with low concentration of beta-lactams (Coque *et al.* 2008). As reported in this work, it is of particular concern the fact that 88.9% of the CTX-M-producers are multiresistant (93.3% P and 66.6% UP). Among CTX-M-producers isolated from polluted waters, resistance to quinolones, aminoglycosides, tetracyclines and the combination sulfamethoxazole-trimethoprim was highly prevalent. Due to their ability to capture and incorporate gene cassettes from the environment, integrons have an important

role on the spread of multidrug resistance in Gram-negative bacteria. In this work, class 1 integrons were detected in 56.4% of ESBL producers (48.7% in P and 7.7% in UP sites).

Analyzing only the cultivable fraction of Gram-negative bacteria in MacConkey agar plates might underestimate the diversity of $bla_{\text{CTX-M}}$ gene variants present in the lotic ecosystem under study. To overcome this methodological aspect, it was applied a culture-independent approach to further analyze the diversity of $bla_{\text{CTX-M}}$ genes in both environments. For that, two clone libraries of $bla_{\text{CTX-M}}$ gene fragments amplified from polluted and unpolluted environmental DNA were constructed and analyzed. In P library the variety of CTX-M-like genes was much higher than in UP library. This probably is related to higher anthropogenic selective pressures posed by the release of antibiotics and/or antibiotic resistant bacteria. Also other studies have shown that other contaminants can also contribute to the persistence of antibiotics resistance in the environment, like for example heavy metals and disinfectants (Martinez 2009, 2009a). Within P library similarity with $bla_{\text{CTX-M}}$ genes from 4 clusters and also with chromosomal variants referred as ancestors of clusters CTX-M-1 and CTX-M-2 was found. Interestingly, the majority of $bla_{\text{CTX-M-like}}$ sequences found in unpolluted DNA were similar to chromosomal class A ESBLs that have been described in *Rahnella* spp. ($bla_{\text{RAHN-1}}$ and $bla_{\text{RAHN-2}}$) and *Serratia fonticola* ($bla_{\text{FONA-5}}$). In a previous work a $bla_{\text{CTX-M}}$ library cloned from urban river sediment DNA presented also high diversity of $bla_{\text{CTX-M}}$ sequences with 13 variants found (Lu *et al.* 2010). Overall, results here presented show clear differences in polluted and unpolluted environments. While in unpolluted rivers we found at most 4 variants with the majority related to ancestor chromosomally located genes, in polluted waters up to 14 variants were found (from 4 out of 5 clusters so far identified in CTX-M enzymes).

A shift in the distribution of different ESBLs has recently occurred in European clinical settings, with a dramatic increase of CTX-M enzymes over TEM and SHV variants. More than 110 CTX-M variants have been described so far. Due to the high homology with chromosomal beta-lactamases from different *Kluyvera* species these are now recognized as CTX-M ancestors, such as KLUA-1 from *K. ascorbata* and KLUG-1 from *K. georgiana* (Cantón and Coque 2006). However the diversity we found in polluted sites cannot be attributed to the presence of bacteria carrying CTX-M ancestral genes. As

in clinics, our results suggest that CTX-M genes dominance is correlated to selective pressures imposed by human activities.

These findings sustain our hypothesis that anthropogenic activities might modulate the environmental resistance gene pool and promote antibiotic resistance dissemination. Also, we have shown that ESBL genes are a form of environmental pollution, either resulting from the intake of ARGs or ARB from human activities or from the selection of environmental resistant bacteria by subtherapeutic antibiotic doses released into the environment. In our study, ESBL genes were found in genera not included in routine evaluation of water quality, associated with the genetic platforms needed for their mobilization and transfer. Thus, we suggest that data on the occurrence and diversity of ESBL genes, and specifically CTX-M genes, can be used to assess ecosystems health and antibiotic resistance evolution. Yet, more studies on other geographical locations are needed to validate this application. These genes are also good candidates to be used as pollution indicators. To further confirm this potential, source tracking approaches must be conducted to link the presence of CTX-M genes to specific sources of contamination.

3.1.5 CONCLUSIONS

The work here presented showed that occurrence and antimicrobial susceptibility profiles of CTX^R bacteria are markedly different between polluted and unpolluted lotic ecosystems; the same happens with occurrence and diversity of clinically relevant ESBL genes. Our results validate the hypothesis that anthropogenic impacts on water environments are modulators of the resistance gene pool and promote dissemination of antibiotic resistance.

In addition, it suggests that $bla_{\text{CTX-M-like}}$ genes may constitute indicators of pollution by antibiotics, useful to study antibiotic resistance dispersal in aquatic environments.

We also conclude that the dissemination of resistance to broad-range antibiotics such as cefotaxime may be at an earlier stage in pristine environments, providing the opportunity to continuing studying the impact of anthropogenic-driven dissemination and evolution.

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SUPPLEMENTAL MATERIAL

TABLE S1: GPS coordinates for the 12 sites under study

Site	River	Coordinates
1	Antuã	40° 44.580 N, 08° 34.173 W
2	Úl	40° 51.114 N, 08° 29.419 W
3	Ínsua	40° 51.070 N, 08° 27.118 W
4	Caima	40° 43.513 N, 08° 06.483 W
5	Zela	40° 43.170 N, 08° 06.440 W
6	Vouga	40° 44.226 N, 08° 05.191 W
7	Alcofra	40° 37.420 N, 08° 11.410 W
8	Alfusqueiro	40° 38.541 N, 08° 16.517 W
9	Águeda	40° 35.478 N, 08° 14.101 W
10	Águeda	40° 34.144 N, 08° 26.509 W
11	Da póvoa	40° 37.304 N, 08° 25.571 W
12	Cértima	40° 30.518 N, 08° 27.533 W

TABLE S2: Physical, chemical and microbiological parameters determined according to Portuguese laws (D.L. 236/98) and water quality classification, for the 12 sites under study.

Sites	pH	Temp. (ºC)	Cond. µS/cm	DO mg/l	TSS mg/l	Nit. mg/l	Clor. mg/l	Phosp. mg/l	Amm. mg/l	COD mg/l	BOD_5 mg/l	Color mg/l PtCo	Smell Dil. factor	TC CFU/100mL	FC CFU/100mL	FS CFU/100mL	Classification
1	7,50	19,7	226.0	9,20	15	30,2	28	1,1	< 0,10	< 30	1,1	28	1	11.1 X 10^4	324	166	Polluted
2	8,70	19,1	251.0	8,70	14	15,8	30	2,7	5,49	36	4,2	75	1	140.4 X 10^4	INC	49500	Polluted
3	7,30	17,7	96.0	10,00	13	14,7	< 25	0,5	0,13	< 30	< 1,0	79	1	54.0 X 10^4	69	334	Unpolluted
4	7,30	20,8	100.0	9,60	16	< 11	< 25	0,8	0,29	< 30	< 1,0	31	1	54.0 X 10^4	648	10	Unpolluted
5	6,94	20,1	69.0	7,84	26	< 11	< 25	0,2	< 0,10	< 30	< 1,0	13	1	1.8 X 10^4	156	286	Unpolluted
6	6,83	23,7	68.0	7,02	< 10	< 11	< 25	0,1	< 0,10	< 30	< 1,0	28	1	12.3 X 10^4	1	174	Unpolluted
7	6,35	19,2	39,9	7,32	< 10	< 11	< 25	< 0,1	< 0,10	< 30	< 1,0	12	1	0.07 X 10^4	8	20	Unpolluted
8	7,03	22,8	52,1	6,75	< 10	< 11	< 25	< 0,1	< 0,10	< 30	< 1,0	19	2	5.5 X 10^4	82	204	Unpolluted
9	6,53	21,0	38,4	6,92	< 10	< 11	< 25	< 0,1	< 0,10	< 30	< 1,0	14	1	0.09 X 10^4	0	42	Unpolluted
10	6,78	21,4	6,6	7,30	< 10	< 11	< 25	< 0,1	< 0,10	< 30	< 1,0	24	1	4.4 X 10^4	73	44	Unpolluted
11	7,57	18,9	722.0	4,89	< 10	< 11	< 25	< 0,1	< 0,10	< 30	< 1,0	13	1	2.7 X 10^4	474	144	Unpolluted
12	7,11	18,5	68,7	7,60	14	19,4	33	1,5	0,23	< 30	1,6	43	1	6.6 X 10^4	626	404	Polluted

Abreviations: Temp., temperature; Cond., conductivity; DO, dissolved oxygen; TSS, total suspend solids; Nit., nitrates; Clor., clorets; Phosp., phosphates; Amm., ammonium; COD, chemical oxygen demand; BOD5, biological oxygen demand; TC, total coliforms; FC, fecal coliforms; FS, fecal streptococci.

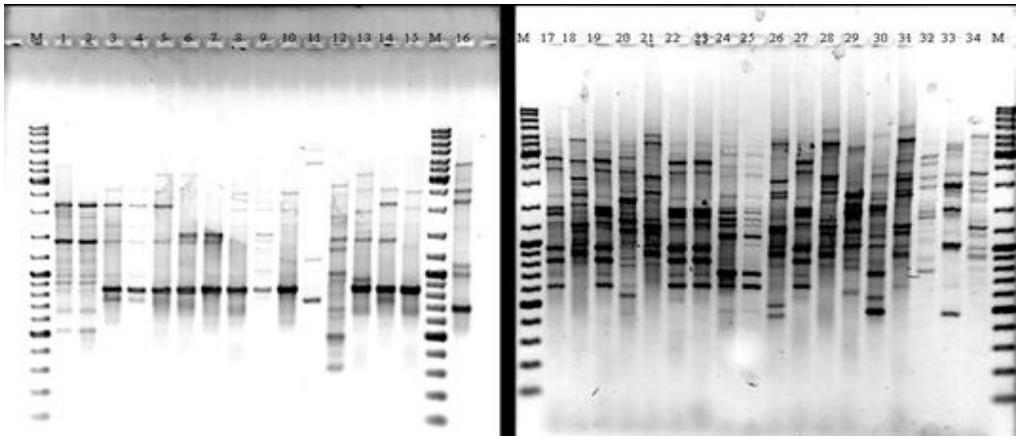

FIG S1: Example of BOX-PCR fingerprints generated by PCR with BOXA1R primer, in 1.5% agarose gels (M- Gene Ruler DNA Ladder Mix, MBI Fermentas, Lithuania; 1-34, cefotaxime resistant isolates obtained from water samples).

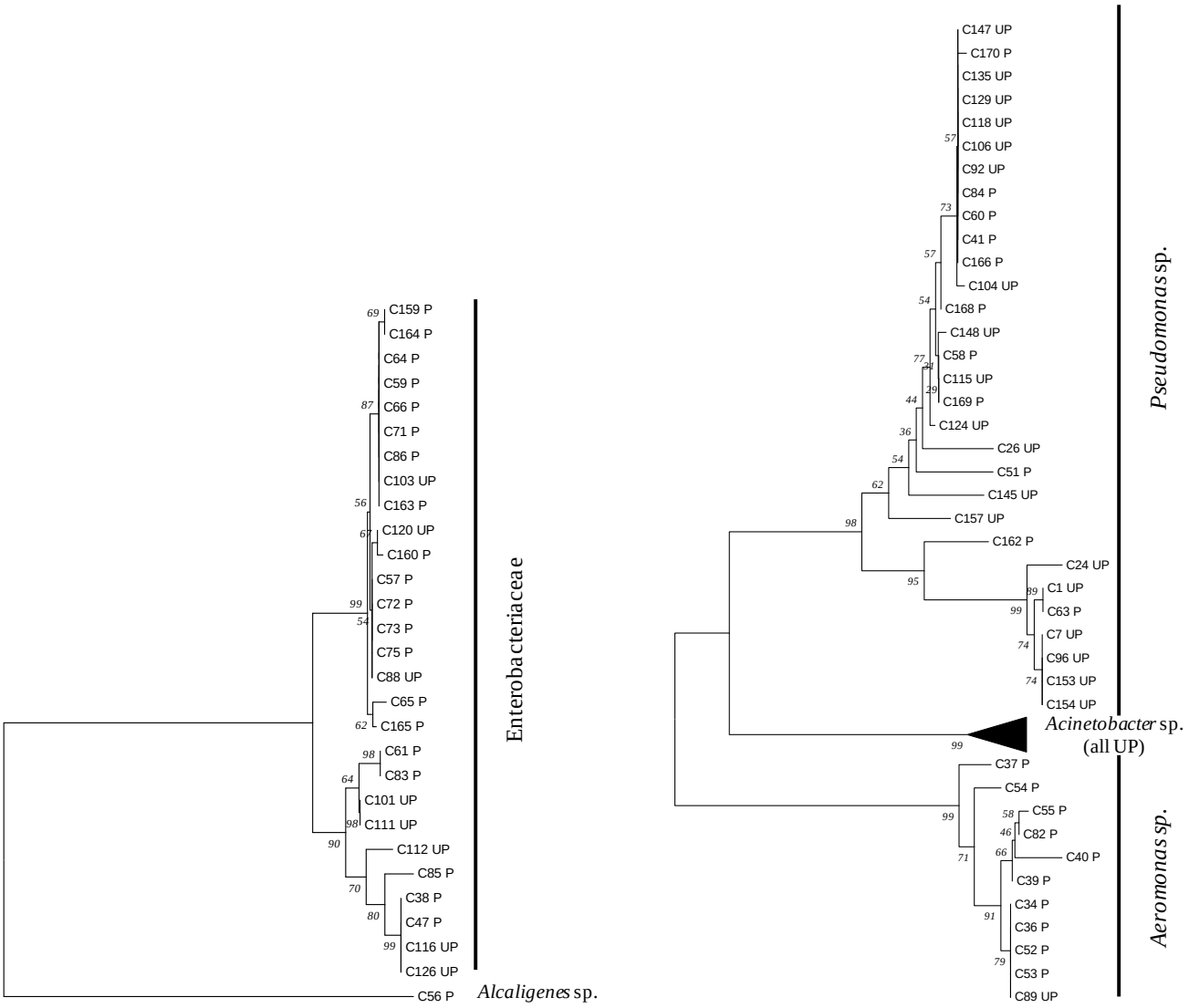

FIG S2: Phylogenetic tree based on 16S rRNA gene sequences of isolates from polluted (P) and unpolluted (UP) rivers; Sequences displaying 100% homology were removed (14P+35UP Pseudomonas sp., 3P Aeromonas sp.) (Left- Enterobacteriaceae and *Alcaligenes* sp.; Right- *Aeromonas* sp., *Pseudomonas* sp. and *Acinetobacter* sp.).

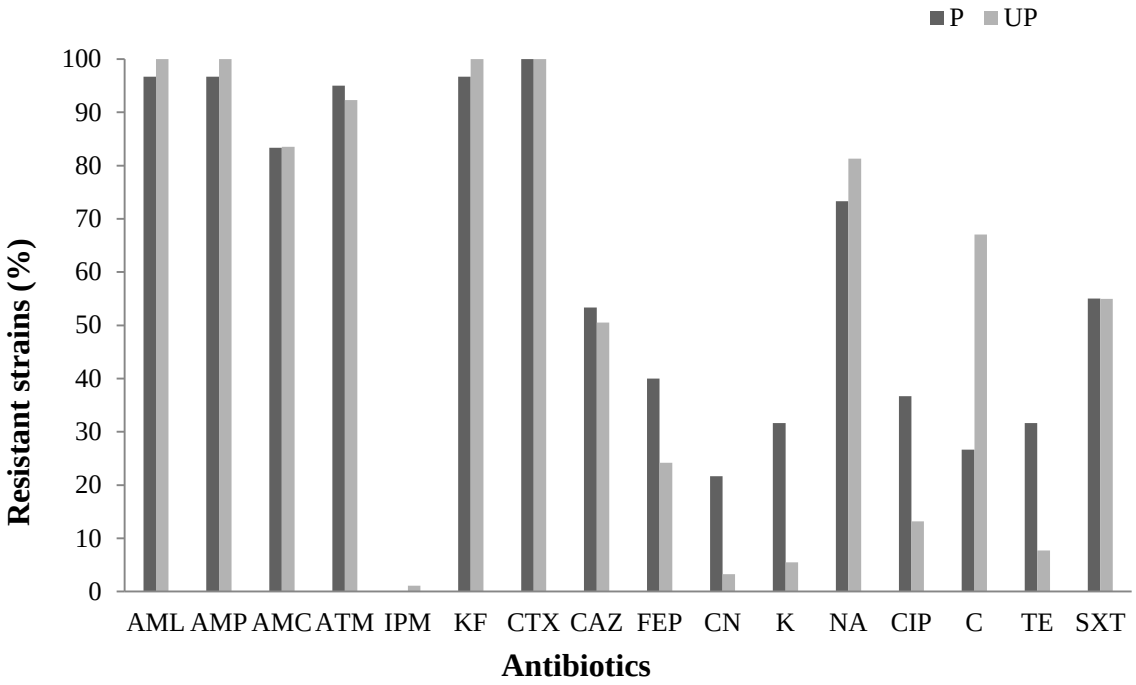

FIG S3: Antimicrobial resistance of isolated strains. AML, amoxicillin; AMP, ampicillin; AMC, amoxicillin/clavulanic acid; ATM, aztreonam; IPM, imipenem; KF, cephalotin; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; CN, gentamicin; K, kanamycin; NA, nalidixic acid; CIP, ciprofloxacin; C, chloramphenicol; TE, tetracycline; SXT, trimethoprim/sulfamethoxazole.

3.2

CO-RESISTANCE TO DIFFERENT CLASSES OF ANTIBIOTICS AMONG ESBL-PRODUCERS FROM AQUATIC SYSTEMS

Abstract

In this study we investigated the co-occurrence of resistance to non-beta-lactams among cefotaxime-resistant extended-spectrum beta-lactamase (ESBL) producers ($ESBL^+$) *versus* non-ESBL producers ($ESBL^-$), from aquatic environments. Higher prevalence of resistance to tetracycline, fluoroquinolones and aminoglycosides were observed in $ESBL^+$. Among $ESBL^+$ resistant to tetracycline (*n*=18), *tet*(A) was detected in 88.9% and *tet*(B) in 16.7%. Among fluoroquinolone-resistant-$ESBL^+$ (*n*=15), *aacA4*-cr and *qnr*VC4 were identified in 26.6% and 40% strains, respectively. The *qnrVC4* gene was detected for the first time in *Pseudomonas* sp. and *Escherichia coli*. Class 1 integrase genes were detected in 56.41% of $ESBL^+$ and in 27.67% $ESBL^-$. Gene cassette arrays identified conferred resistance to aminoglycosides (*aadA*-type genes and *aacA4*), trimethoprim (*dfrA17*), chloramphenicol (*catB8*), fluoroquinolones (*qnrVC4*) and beta-lactams (bla_{OXA-10}). Conjugation experiments were performed with CTX-M-producers. Transconjugants showed multiresistance to 3 or more classes of antibiotics, and conjugative plasmids were assigned to IncF, IncK and IncI1 replicons. Results obtained showed that co-selection of resistance to aminoglycosides, quinolones and tetracyclines is prevalent among ESBL-producers and that these features are successfully mobilized by IncF, IncK and IncI1 conjugative plasmids. This study reinforces the importance of natural aquatic systems as reservoir of mobile genetic platforms carrying multiple resistance determinants. Moreover, to the best of our knowledge, this constitutes the first observation of IncK::CTX-M-3 in *Aeromonas hydrophila* and the first report of IncK plasmids in Portugal.

3.2.1 INTRODUCTION

Antibiotic resistance is no longer seen as restricted to clinical settings but as ubiquitous ecological phenomena (Laroche *et al.* 2009). Aquatic systems, such as rivers and streams, constitute important antibiotic resistance reservoirs (Lupo *et al.* 2012) where anthropogenic pressures may promote the dissemination of antibiotic resistance genes and bacteria (Tacão *et al.* 2012). Co-resistance is the outcome of the accumulation of resistance mechanisms to different classes of antibiotics on the same bacterial strain; this happens by means of mutation or acquisition of novel resistance genes by horizontal transfer.

Multidrug resistance is a comprehensive feature, including resistance to compounds such as heavy metals or disinfectants, in addition to antibiotics (Skipppington *et al.* 2011). Different determinants of resistance may be linked, carried by mobile genetic platforms like plasmids, transposons or integrons (Woodford *et al.* 2011), with plasmids playing a central role in the dissemination of resistance genes by horizontal transfer. Multidrug resistant strains can be selected by a single antibiotic but also by the exposure to different compounds (Canton and Ruiz-Garbajosa 2011).

Extended-spectrum beta-lactamases (ESBLs) are capable of hydrolyzing penicillins, cephalosporins and also the monobactam aztreonam. When dealing with bacterial infections caused by ESBL-producers ($ESBL^+$), a multiresistance phenotype clearly limits the therapeutic options (van Hoek *et al.* 2011). Plasmids carrying ESBL genes frequently are conjugative and lodge determinants of resistance to non-beta-lactams such as tetracyclines, quinolones or aminoglycosides. In that case, ESBL-positive strains are multiresistant and pose major public health concerns (Carattoli *et al.* 2011, Coque *et al.* 2008a).

Aquatic systems such as rivers are exposed to disposals from different sources, receiving chemical and microbial contaminants of industrial, agricultural and domestic origins. Water pollution was shown to modulate the antibiotic resistome (Tacão *et al.* 2012) and aquatic environments may act as reactors with incubation conditions that promote genetic exchanges and contribute to the spread of antibiotic resistance (Lupo *et al.* 2012).

In a previous work, we analyzed a set of cefotaxime-resistant strains isolated from river waters in Portugal (Tacão *et al.* 2012): multiresistance was frequent among $ESBL^+$ strains, mostly carrying $bla_{CTX\text{-}M}$.

The present investigation was conducted to evaluate which resistance genes are co-selected with ESBL genes in aquatic systems and to what extent are those genes included in linkage groups carried by mobile genetic elements. For that, we analyzed the prevalence of antibiotic resistance traits among $ESBL^-$ and $ESBL^+$ strains and tested their association to conjugative plasmids and integrons.

3.2.2 MATERIALS AND METHODS

3.2.2.1 Bacterial strains

In this study, we analyzed 151 cefotaxime-resistant Gram-negative strains previously isolated from surface waters of 12 rivers located in Portugal (Tacão *et al.* 2012). Of these strains, 39 were identified as ESBL-producers ($ESBL^+$) and 112 as ESBL-non producers ($ESBL^-$). The phylogenetic affiliation of bacterial strains used in this study is presented in Table 1.

3.2.2.2 Antibiotic susceptibility profiles and ESBL production

The disc diffusion method on Mueller-Hinton agar was used to test antibiotics from 6 classes: beta-lactams (penicillins, monobactams, 3rd and 4th generation cephalosporins and carbapenems), quinolones, aminoglycosides, phenicols, tetracyclines and the combination sulfamethoxazole/trimethoprim. The discs (Oxoid, Basingstoke, UK) contained the following antibacterial agents and concentrations according to the Clinical Laboratory Standards Institute guidelines (CLSI 2012): amoxicillin (10 mg), amoxicillin/clavulanic acid (20 mg/10 mg), ampicillin (10 mg), aztreonam (30 mg), cefepime (30 mg), ceftazidime (30 mg), ciprofloxacin (5 mg), chloramphenicol(30 mg),

gentamicin (10 mg), imipenem (10 mg), kanamycin (30 mg), nalidixic acid (30 mg), sulfamethoxazole/trimethoprim (25 mg) and tetracycline (30 mg). As quality control, E. coli ATCC 25922 was used. Detection of ESBL was carried out by a clavulanic acid combination disc method, based on comparison of the inhibition zones of discs (Oxoid, UK) of cefpodoxime (10 mg) and cefpodoxime-plus-clavulanate (10 þ 1 mg). The organisms were classified as sensitive, intermediate, or resistant according to the Clinical Laboratory Standards Institute guidelines (CLSI 2012), after 24 h of incubation at 37 ºC.

TABLE 1: Bacterial strains used in this work.

ESBL-production	Phylogenetic affiliation	No. of isolates
ESBL $^{+}$	*Aeromonas* sp.	9
(*n*=39)	*Escherichia coli*	14
	Acinetobacter sp.	2
	Pseudomonas sp.	14
ESBL $^{-}$	*Aeromonas* sp.	10
(*n*=112)	*Escherichia coli*	4
	Acinetobacter sp.	17
	Pseudomonas sp.	69
	Enterobacter sp.	10
	Citrobacter freundii	1
	Alcaligenes sp.	1

3.2.2.3 PCR amplification of resistance determinants

Genetic determinants of resistance to tetracycline [*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G) and *tet*(M)] and fluoroquinolones (*parC* and *gyrA* mutations, *qnrA*, *qnrB*, *qnrS*, *qnrVC*, *qepA* and *aacA4-cr)* were inspected by PCR using previously described primers and conditions (see supplemental material Table S1). Amplification of *qnrVC* genes was carried out by PCR using primers designed in this study (qnrVC_F: 5’ -

GGATAAAACAGACCAGTTATATGTACAAG – 3' and qnrVC_R: 5'- AGATTT GCGCCAATCCATCTATT -3'). Amplicons were confirmed by DNA sequencing.

3.2.2.4 Integron screening and characterization

The presence of integrons was assessed through PCR amplification of *intI1*, *intI2* and *intI3* integrase genes (supplemental material Table S1). The variable regions of integrase-positive strains were further amplified by PCR using several combinations of primers (supplemental material Table S1) and sequenced.

3.2.2.5 Conjugation experiments

Eighteen strains containing $bla_{\text{CTX-M}}$ genes (Tacão *et al.* 2012) were used as donors in mating assays, from which: 3 *Aeromonas hydrophila* carrying $bla_{\text{CTX-M-3}}$, 9 *E. coli* carrying $bla_{\text{CTX-M-15}}$, 3 *E. coli* carrying $bla_{\text{CTX-M-1}}$, 1 *E. coli* carrying $bla_{\text{CTX-M-14}}$, 1 *E. coli* carrying $bla_{\text{CTX-M-32}}$ and 1 *Pseudomonas* sp. with $bla_{\text{CTX-M-15}}$). The azide-resistant *E. coli* J53 was used as recipient strain. Transconjugants were selected in Luria-Bertani agar plates (LA) supplemented with azide (100 µg/ml) and cefotaxime (8 µg/ml). Transconjugants were verified by ERIC-PCR (Versalovic *et al.* 1994) to confirm the identity of the host and, to confirm plasmid acquisition, the detection of $bla_{\text{CTX-M}}$ gene was performed as previously described (Tacão *et al.* 2012). Primers used are listed in supplemental material Table S1.

3.2.2.6 Transconjugants analysis

Plasmid DNA from transconjugants was purified using the Qiagen Plasmid Mini-kit (Qiagen GmbH, Germany). Molecular diversity of plasmids was examined by restriction analysis with *Pst*I and *Bst*1770I (Fermentas, Lithuania) as described elsewhere (Moura *et*

al. 2012b). Antimicrobial susceptibility profiles of transconjugants and recipient strains were determined as described above.

3.2.2.7 Replicon typing

Detection of IncA/C, IncB/O, IncF (FIA, FIB, FIC, FIIA, FrepB subgroups), IncHI1, IncHI2, IncI1-Iγ, IncK, IncL/M, IncN, IncP IncT, IncW and IncY replicons was performed by PCR, using primers (see Table S1, supplemental material) and conditions as described previously (Moura *et al.* 2012b). For confirmation, the nucleotide sequence of the amplicons was determined. For transconjugants that received more than one plasmid, the location of CTX-M was clarified by southern blot hybridization, as previously described (Henriques *et al.* 2006).

3.2.2.8 Statistical Analysis

Statistical analysis was performed by two-sample *t*-test.

3.2.2.9 Nucleotide sequence accession numbers

Nucleotide sequences were deposited in GenBank under the accession numbers: JQ837985-JQ838002 (gene cassette arrays) and JQ838003-JQ838009 (*qnrVC*).

3.2.3 RESULTS

3.2.3.1 ESBL$^+$ vs. ESBL$^-$ antibiotic resistance profiles

Antibiotic resistance profiles were determined for 39 ESBL$^+$ (26%) and 112 ESBL$^-$ (74%) cefotaxime-resistant strains. Results are shown in Figure 1.

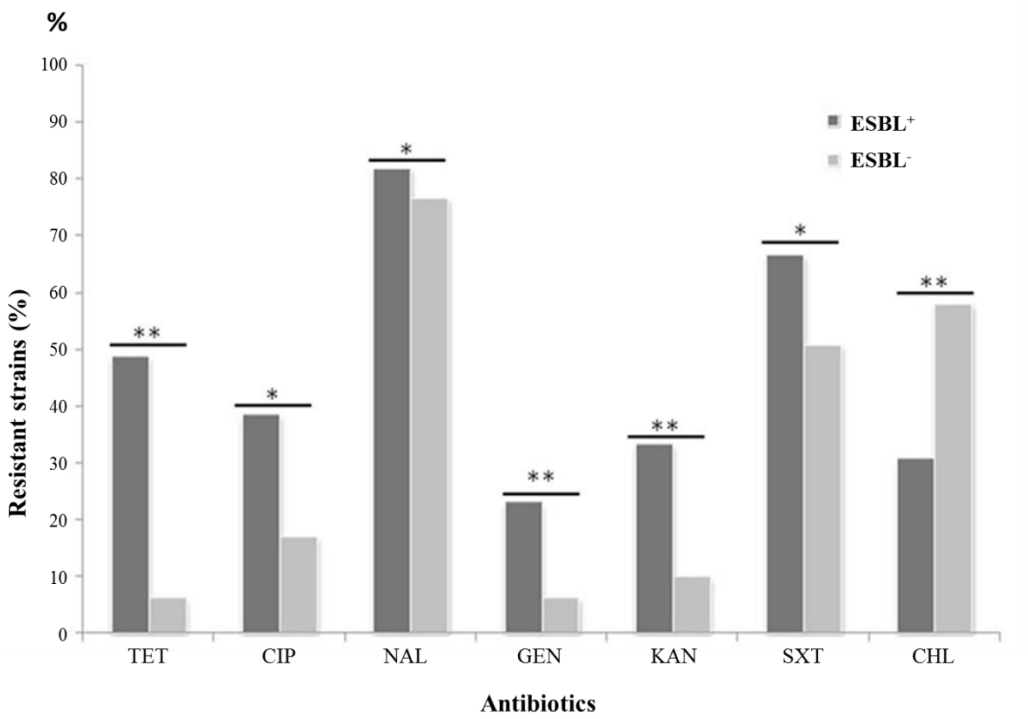

FIG.1: Prevalence of resistant strains (%) among ESBL-producers ($ESBL^+$) and non-ESBL-producers ($ESBL^-$), to tetracycline (TET), quinolones (NAL, nalidixic acid; CIP, ciprofloxacin), aminoglycosides (GEN, gentamicin; KAN, kanamycin), trimethoprim/sulfamethoxazole (SXT) and chloramphenicol (CHL). Statistical significance is shown with $p<0.05$ (*) and $p<0.01$ (**).

Multidrug resistance was slightly higher among ESBL producers (79.5% against 71.4%). Resistance to tetracycline was significantly more prevalent in $ESBL^+$ strains (48.7% vs. 6.3%, $p<0.05$). Significant differences ($p<0.05$) were also obtained for resistance to quinolones (ciprofloxacin, 38.5% vs. 17.0%; nalidixic acid, 82.1% vs. 76.8%), aminoglycosides (kanamycin, 33.3% vs. 9.8%; gentamicin, 23.1% vs. 6.3%) and the combination sulfamethoxazole-trimethoprim (66.7% vs. 50.9%). Only for chloramphenicol an opposite trend was observed being resistance significantly more prevalent in $ESBL^-$ strains (30.8% vs. 58.0%, $p< 0.05$) (FIG. 1).

3.2.3.2 Integron screening and characterization

The prevalence of *intI1* was 56.41% (22 out of 39) among $ESBL^+$ strains and 27.67% (31 out of 112) among $ESBL^-$ strains (Table 2).

Integron variable regions were characterized in 41% of the *intI*-positive strains. These included nine different gene cassette arrays: *aadA1* (*Pseudomonas* sp., *Aeromonas* sp.), *aadA2* (*A. hydrophila*), *aadA16/aacA4'* fusion (*A. hydrophila*), *aadA6 – orfD* (*Pseudomonas* sp.), *catB8 – aadA1* (*A. hydrophila*), *dfrA1 – aadA1* (*E. coli*), *dfrA17 – aadA5* (*E. coli, Aeromonas* sp., *Pseudomonas* sp.), *qnrVC4 – aacA4'-17* (*E. coli, A. hydrophila*), *blaOXA-10 - aacA4'* (*A. hydrophila*). Empty integrons were detected in *A. hydrophila, Aeromonas* sp., *Citrobacter freundii* and *Pseudomonas* sp.. Among $ESBL^+$ strains, the gene cassette array *dfrA17-aadA5* was the most frequently detected (Table 2).

The simultaneous presence of two integrons with different gene cassette arrays was observed in 4 strains: *A. hydrophila* C52 $ESBL^-$ (*catB8 –aadA1*and bla_{OXA-10} - *aacA4'*), *A. hydrophila* E1 $ESBL^+$ (empty integron and *aadA16 /aacA4'*), *E. coli* C88 $ESBL^+$ (*dfrA17 – aadA5* and *qnrVC4 – aacA4'-17*) and *A. hydrophila* C89 $ESBL^+$ (*aadA2* and *qnrVC4 – aacA4'-17*). Sequence analysis revealed the presence of PcH1 (n=14) and PcW (n=3) promoter variants, responsible for the expression of gene cassettes, well as of internal cassette-specific promoters $P_{qacE\Delta1}$ and P_{qnrVC} (in arrays *aaA16-aacA4'* and *qnrVC4-aacA4'-17*, respectively).

3.2.3.3 Tetracycline resistance genetic determinants

Among tetracycline-resistant strains (n=26), *tet*(A) was the most frequently detected resistance determinant, present in 88.9% $ESBL^+$ (16/18) and 50% $ESBL^-$ (4/8). The *tet*(B) gene was detected only in $ESBL^+$ strains, in 16.7% (3/18). In one $ESBL^+$ strain both *tet*(A) and *tet*(B) genes were detected. No *tet*(C), *tet*(D), *tet*(E), *tet*(G) or *tet*(M) were detected.

TABLE 2: Prevalence of different gene cassette arrays identified among class 1 integrons detected in ESBL$^+$ and ESBL$^-$ strains.

ESBL production	*intI1*	Gene cassette arrays[a]	Prevalence (no. of isolates)	Phylogenetic affiliation[b] (no. of isolates)
ESBL $^+$	56.41% (22/39)	*dfrA17 – aadA5*	22.72% (5)	A (1), Ec(3), P (1)
		aadA1	4.54% (1)	A
		qnrVC4 – aacA4'-17	9.09% (2)	Ah, Ec
		aadA16 /aacA4'	4.54% (1)	Ah
		aadA2	4.54% (1)	Ah
		Empty integron	13.63% (3)	Ah
		n.d.*	54.54% (12)	A (1), Ec (9), P (2)
ESBL $^-$	27.67% (31/112)	*catB8 –aadA1*	3.22% (1)	Ah
		bla_{OXA-10} - *aacA4'*	3.22% (1)	Ah
		dfrA17 – aadA5	3.22% (1)	Ec
		aadA1	3.22% (1)	P
		dfrA1 - aadA1	3.22% (1)	Ec
		aadA6 – orfD	3.22% (1)	P
		Empty integron	9.67% (3)	A, Cf, P
		n.d.*	74.19% (23)	Ac (5), E (1), Ec (1), P (16)

[a] n.d.: not determined; [b] A- *Aeromonas* sp., Ah – *Aeromonas hydrophila*, Ac- *Acinetobacter* sp., Cf- *Citrobacter freundii*, E- *Enterobacter* sp., Ec- *E. coli*, P- *Pseudomonas* sp..

3.2.3.4 Fluoroquinolone resistance genetic determinants

The *qnrA*, *qnrB*, *qnrS* and *qepA* genes were not detected among the fluoroquinolone-resistant strains (*n*=34) (Table 3).

The *aacA4-cr* gene was detected in 26.6% ESBL$^+$ (4/15) and 36.84% ESBL$^-$ (7/19). The *qnrVC4* gene was identified in 40% ESBL$^+$ (6/15) and 15.7% ESBL$^-$ (3/19), in *Pseudomonas* sp. (*n*=4), *Aeromonas* sp. (*n*=4) and *E. coli* (*n*=1) (FIG. 2).

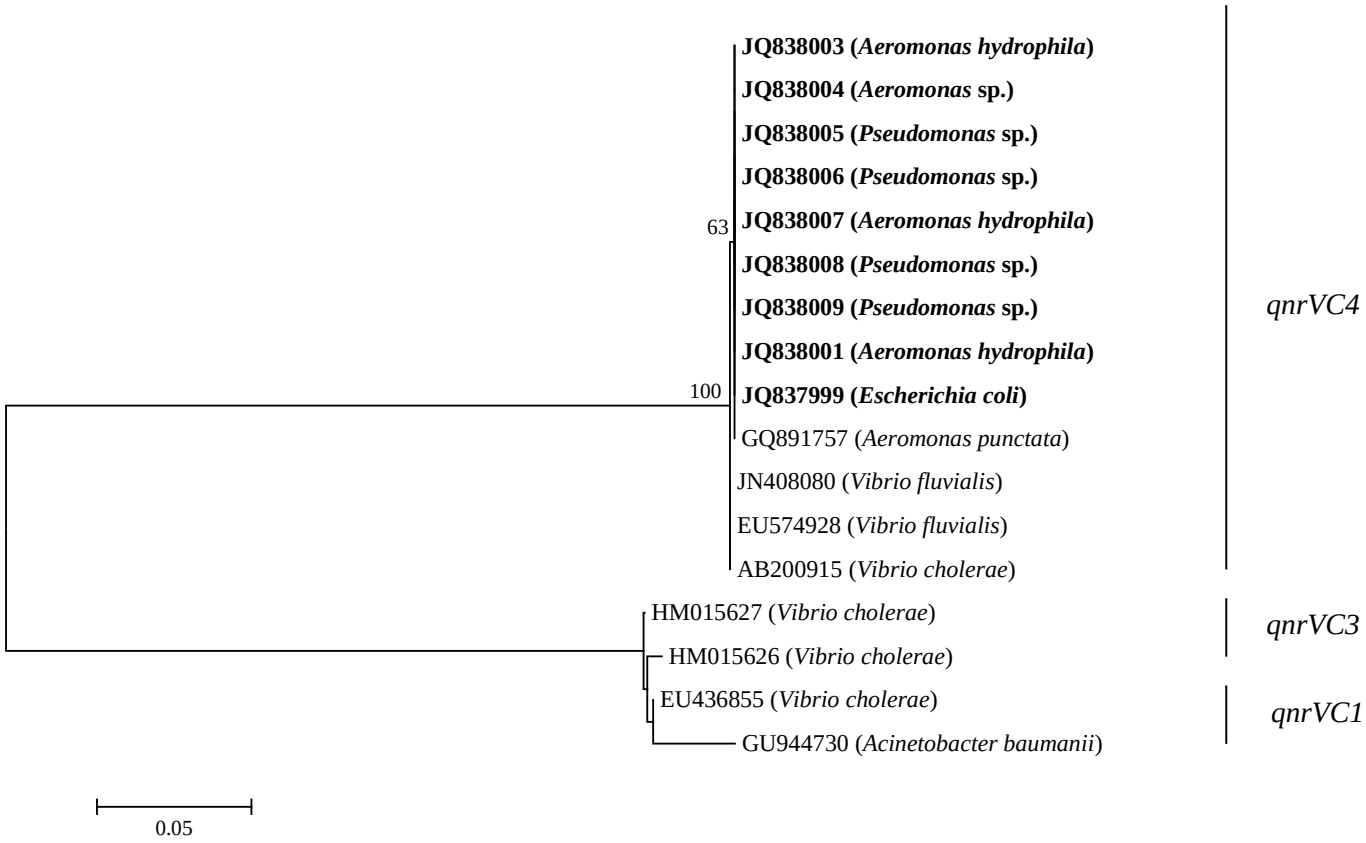

FIG. 2: Phylogenetic tree of *qnrVC* genes. Accession numbers and phylogenetic affiliation are indicated. Sequences obtained in this study are shown in bold.

Four ESBL$^+$ and 1 ESBL$^-$ strains presented both the *qnrVC4* and *aacA4-cr* genes. Regarding chromosomal-encoded resistance, no mutations were identified in DNA gyrase gene (*gyrA*). However, in 13 out of 34 strains one or two mutations in the topoisomerase IV gene (*parC*) were identified. Of these, a Ser80Ile mutation was identified in a non-ESBL-producer *E. coli* strain. The remaining 12 were found in ESBL$^+$ strains with *parC* (80,84) in *Aeromonas* sp. (*n*=2), *Pseudomonas* sp. (*n*=1), *E. coli* (*n*=5) and *parC*(80) in *E. coli* strains (*n*=2). None of the fluoroquinolone resistance mechanisms searched was identified in 10 ESBL$^-$ strains (Table 3).

TABLE 3: Prevalence of different fluoroquinolones-resistance mechanisms identified among $ESBL^+$ and $ESBL^-$ strains.

ESBL-production on fluoroquinolones-resistant strains	Resistance mechanism detected	Prevalence (no. of isolates)	Phylogenetic affiliation[a] (no. of isolates)
$ESBL^+$ (*n*=15)	*parC*(80)	20.00% (3)	Ec
	parC(80,84)	40.00% (6)	Ah (1), Ec (5)
	qnrVC4	6.66% (1)	Ah
	qnrVC4 and *aacA4-cr*	13.33% (2)	Ah
	qnrVC4 and *parC*(80)	6.66% (1)	P
	qnrVC4, *aacA4-cr* and *parC*(80)	6.66% (1)	Ec
	qnrVC4, *aacA4-cr* and *parC*(80,84)	6.66% (1)	A
$ESBL^-$ (*n*=19)	*aacA4-cr*	26.31% (5)	A (1), Ac (1), P (3)
	qnrVC4	10.52% (2)	P
	qnrVC4 and *aacA4-cr*	5.26% (1)	P
	aacA4-cr and *parC*(80)	5.26% (1)	Ec
	Unknown	52.63% (10)	Ac (3), Ah (1), E (3), P (3)

a A- *Aeromonas* sp., Ah -*Aeromonas hydrophila*, Ac- *Acinetobacter* sp., E- *Enterobacter* sp., Ec- *E. coli*, P- *Pseudomonas* sp.).

3.2.3.4 Analysis of CTX-M-*transconjugants*

Six out of 18 donor strains generated transconjugants resistant to azide and cefotaxime (Table 4).

Restriction analysis of transconjugants revealed 7 different profiles (strain E1 generated 2 transconjugants with distinct plasmid content). Conjugative plasmids detected were assigned to replicons FrepB, FIB, K and I1. One transconjugant could not be assigned to any of the replicon types tested. In two transconjugants more than one plasmid type was present: FrepB and FIB (1T) and FrepB and IncK (1aT).

The $bla_{CTX\text{-}M}$ gene amplified from plasmid DNA purified from all the transconjugants. The $bla_{CTX\text{-}M}$ genes transferred were: $bla_{CTX\text{-}M\text{-}1}$ in IncI1 (from *E. coli* donor strains); $bla_{CTX\text{-}M\text{-}3}$ in IncF and IncK (from *A. hydrophila*); and $bla_{CTX\text{-}M\text{-}15}$ in IncI1 (from *E. coli* and *Pseudomonas* sp. strains). As expected, all transconjugants displayed phenotypes of

resistance to 3rd and 4th generation cephalosporins and monobactams and were positive for ESBL production, in contrast to the recipient strain. In addition, the majority of transconjugants were resistant to penicillins (ampicillin and amoxicillin, 6 out of 7 transconjugants) and to the combination sulfamethoxazole/trimethoprim (5 out of 7) (Table 4). Resistance to carbapenems, quinolones, aminoglycosides, tetracyclines and phenicols was also observed. In three cases the transferred plasmids conferred multiresistance phenotypes: transconjugant 1T was resistant to beta-lactams, aminoglycosides and tetracycline, transconjugant 1aT was resistant to beta-lactams, aminoglycosides, tetracycline and phenicols and transconjugant 4T was resistant to beta-lactams, aminoglycosides, tetracycline, quinolones and sulfamethoxazole/trimethoprim.

TABLE 4: Antibiotic resistance profile and replicon types of transconjugants carrying bla_{CTX-M} genes.

Donor strain	**CTX-M gene**	**Transconjugant**	**Resistance phenotype of transconjugant**[a]	**Incompatibility group**
A. hydrophila E1	CTX-M-3	1T	CPD, CAZ, FEP, ATM, TET, GEN, KAN	FrepB, FIB
A. hydrophila E1	CTX-M-3	1aT	AMP, AML, CPD, FEP, ATM, TET, GEN, CHL	FrepB, K
E. coli E7	CTX-M-15	4T	AMP, AML, CPD, FEP, IPM, ATM, TET, KAN, CIP, NAL, SXT	n.d.[b]
E. coli E18	CTX-M-15	11T	AMP, AML, CPD, CAZ, FEP, ATM, SXT	I1
E. coli E24	CTX-M-1	13T	AMP, AML, CPD, CAZ, FEP, ATM, SXT	I1
E. coli E26	CTX-M-1	14T	AML, CPD, CAZ, FEP, ATM, SXT	I1
Pseudomonas sp. E39	CTX-M-15	18T	AMP, AML, CPD, FEP, IPM, ATM, SXT	I1

[a] AML- amoxicillin, AMP- ampicillin, ATM- aztreonam, CAZ- ceftazidime, CPD- cefpodoxime, FEP- cefepime, GEN- gentamicin, IPM- imipenemo, KAN- kanamycin, NAL- nalidixic acid, SXT- sulfamethoxazole/trimethoprim, TET- tetracycline; [b] n.d.: not determined.

3.2.4 DISCUSSION

Multidrug resistant strains can result from the co-selection of several resistance genes in the same genetic platform or from cross-resistance due to expression of a mechanism responsible for resistance to different compounds (Laroche *et al.* 2009). In this study we analyzed co-resistance among $ESBL^-$ and $ESBL^+$ environmental strains.

Epidemiological studies have shown that ESBL-producers from clinical environments are often multiresistant (Coque *et al.* 2008a). As an example of a worldwide disseminated ESBL is the CTX-M-15 beta-lactamase. Its huge success is due to the association of the $bla_{CTX\text{-}M}$ gene to conjugative plasmids that often harbor genetic determinants of resistance to several classes of antibiotics others than beta-lactams, such as aminoglycosides, fluoroquinolones and tetracyclines (Coque *et al.* 2008a, Perez *et al.* 2007).

Our results showed that ESBL-producers isolated from river waters presented higher levels of resistance to non-beta-lactams, especially to tetracyclines, aminoglycosides and fluoroquinolones. Also the majority were multidrug resistant and harbored class 1 integrons. Resistance to the above mentioned classes of antibiotics has been observed for $ESBL^+$ strains isolated in clinical setting but also in environmental ESBL-producers (Chen *et al.* 2010, Coque *et al.* 2008a). The majority of the $ESBL^+$ strains used in this study were isolated from polluted rivers highly impacted by different human activities (domestic, industrial and agricultural origins) (Tacão *et al.* 2012) which in turn may potentiate the exchange of genetic material and the spread of multiresistant strains.

Moreover, the high prevalence of *intI1*-genes and the presence of identical arrays in different strains suggest that integrons are exchanged and disseminated easier among $ESBL^+$ strains. Although integrons are not considered *per se* as mobile genetic elements, their location on plasmids and transposons gives them the possibility to pass multiresistance traits in a single event (Lupo *et al.* 2009). The gene cassettes identified in this work conferred resistance to aminoglycosides, trimethoprim, chloramphenicol, fluoroquinolones and beta-lactams. The gene cassette array *dfrA17-aadA5* was the most frequently detected among $ESBL^+$. This array has been frequently reported worldwide in both clinical and environmental samples (http://integrall.bio.ua.pt; Moura *et al.* 2009). Moreover, *dfrA17-aadA5* was also described as the most prevalent array among ESBL-

producers isolated from the Yangtze River, China (Chen *et al.* 2010). In integrons detected in both $ESBL^+$ and $ESBL^-$ groups, the control of gene cassette expression was associated to weak PcW and PcH1 variants. Since weaker Pc variants are correlated with higher integrase gene expression and activity (Jové *et al.* 2010), leading to shorter and less stable arrays, these results suggest the existence of a dynamic gene cassette pool in these environments due to high rates of gene cassette recombination. Similar trends have also been reported in integrons from wastewaters and clinical environments, as recently discussed (Moura *et al.* 2012a).

CTX-M beta-lactamases are commonly referred as the most widespread ESBLs nowadays (Coque *et al.* 2008a). Several mechanisms have been associated to its success such as the association to ISEcp1 and ISCR1 insertion sequences (Canton and Coque 2006, Pfeifer *et al.* 2010, Poirel *et al.* 2012a, Tacão *et al.* 2012) and conjugative plasmids belonging to IncF, IncA/C, IncL/M, IncN, IncHI2, IncN, IncI1 and IncK groups, that often carry other antibiotic resistance genes (Canton and Coque 2006, Carattoli, 2009). In the present study, results from the conjugation experiments showed that the multiresistance phenotype registered for *bla*$_{\text{CTX-M}}$ -producers was due to the presence of narrow host range (NHR) plasmids carrying several genetic determinants of resistance. Although NHR plasmids have difficulties in replicating in distantly related hosts (van Hoek *et al.* 2011), both *A. hydrophila* and *Pseudomonas* sp. generated transconjugants using *E. coli* J53 as recipient strain. Plasmids successfully transferred were assigned to IncF::*bla*$_{\text{CTX-M-3}}$, IncK::*bla*$_{\text{CTX-M-3}}$, IncI1::*bla*$_{\text{CTX-M-15}}$ and IncI1::*bla*$_{\text{CTX-M-1}}$.

The *bla*$_{\text{CTX-M-15}}$ gene is the most successfully disseminated *bla*$_{\text{CTX-M}}$ gene and it has been mostly associated to the IncF-family but also to IncL/M, IncA/C, IncN and IncI1 plasmids, as also *bla*$_{\text{CTX-M-3}}$ genes. The *bla*$_{\text{CTX-M-1}}$ has been detected also in FII variants, and in IncL/M, IncN and IncI1 plasmids (Carattoli 2009, Poirel *et al.* 2012a). In this work we identified also an IncK plasmid carrying a *bla*$_{\text{CTX-M-3}}$ gene, as confirmed by southern blot hybridisation. The *bla*$_{\text{CTX-M}}$ gene that has been mostly associated to this incompatibility group is the *bla*$_{\text{CTX-M-14}}$, but also *bla*$_{\text{CTX-M-9}}$ and *bla*$_{\text{CTX-M-10}}$ (Carattoli 2009, Valverde *et al.* 2009). As far as we know the occurrence of IncK plasmids carrying CTX-M genes have never been described in Portugal. Also, to the best of our knowledge, this is the first work reporting IncK::*bla*$_{\text{CTX-M-3}}$ in *A. hydrophila*. One transconjugant was not assigned to any replicon type screened in this study. The antibiotic susceptibility pattern of

this transconjugant revealed a complex resistance phenotype against 5 classes of antibiotics: beta-lactams (penicillins, 3rd and 4th generation cephalosporins, carbapenems, and monobactam), tetracyclines, quinolones, aminoglycosides and the combination of sulfamethoxazole-trimethoprim. Hence, it should be of major interest to fully-sequence and analyze its accessory genes.

In this work we aimed also to understand the resistance mechanisms associated to tetracyclines and fluoroquinolone resistance, in both $ESBL^+$ and $ESBL^-$ strains. Although tetracycline use has been restricted in several countries, tetracycline resistance mechanisms persist (Lupo *et al.* 2012). Our data showed that *tet*(A) and *tet*(B) were the most frequently detected resistance determinant among tetracycline resistant strains. These efflux genes have been frequently detected in the same phylogenetic groups as in this work, which include *Pseudomonas* sp., *Aeromonas* sp. and *Escherichia coli* strains (Roberts *et al.* 2012, van Hoek *et al.* 2010) and also in aquatic systems (Tao *et al.* 2010, Zhang *et al.* 2009). As for fluoroquinolone resistance, three different resistance mechanisms were identified among $ESBL^+$ and $ESBL^-$ strains: amino acid substitutions in quinolones targets (*par*C), enzymatic inactivation (*aacA4-cr*) and alterations in expression levels of quinolones targets (*qnr*VC4). In two $ESBL^+$ strains three different mechanisms were identified, but most presented only one. The acetyltransferase gene *aacA4-cr* was found in nearly one third of strains resistant to fluoroquinolones, in both $ESBL^+$ and $ESBL^-$. Besides being able to acetylate aminoglycosides like kanamycin, amikacin and tobramycin, this variant of the well-disseminated *aacA4*, also acetylates ciprofloxacin, giving an advantage to these strains by conferring resistance to 2 classes of antibiotics (Poirel *et al.* 2012b). Association of *aacA-4-cr* with ESBLs (Rodriguez-Martinez *et al.* 2011) have been reported, for instance in an *E. coli* CTX-M-15-producing strain described in Portugal (Coque *et al.* 2008b).

Surprisingly, in this study the most prevalent variant of the *qnr* gene was *qnrVC4*. Until now, *qnrVC* had only been detected in a few studies in *Vibrio cholerae* (*qnrVC1*, *qnrVC2* and *qnrVC3*), *Acinetobacter* sp. (*qnrVC*-like) and in *Aeromonas punctata* (*qnrVC4*), associated with integrons (*qnrVC1*, *qnrVC-like* and *qnrVC4*) and to a chromosomal integrative conjugative element (*qnrVC3*) (Fonseca *et al.*, 2008; Kim *et al.* 2010, Wu *et al.* 2012, Xia *et al.* 2010). As far as we know, this was the first time that *qnrVC* genes were detected in *Pseudomonas* sp. and *E. coli* strains. Moreover, only 2 out

of 9 *qnrVC4* genes were associated with integrons, suggesting its association with other genetic elements. All deduced amino acid sequences identified in this work were 100% homologous to QnrVC4 (accession no. ADI55014). The majority of the strains harboring *qnrVC4* genes were isolated from polluted water samples (8 out of 9 strains).

3.2.5 CONCLUSIONS

This work highlights the problem associated to multidrug resistant $ESBL^+$ strains that pose an extra concern since it obviously implies limited therapeutic options. Our data has shown that, as in clinical settings, environmental ESBL-producers are often multiresistant and that is a result of co-selection mechanisms such as co-resistance (several resistance mechanisms in the same genetic platform) and cross-resistance (same resistance mechanism for different antibiotics). Integrons and NHR plasmids largely contributed to multiresistance among ESBL producers.

This reinforces the hypothesis that aquatic systems, especially when pressured by anthropogenic activities, may act as reservoirs of resistance genes, potentiating the dissemination and mobilization of genetic platforms that include several resistance determinants, leading to complex phenotypes that may persist even in the absence of antibiotics.

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SUPPLEMENTAL MATERIAL

TABLE S1: Primers used in this study

Primer name	Sequence (5'-3')	Reference
qnrA-F	TTC TCA CGC CAG GAT TTG	1
qnrA-R	CCA TCC AGA TCG GCA AA	1
qnrB-F	GGM ATH GAA ATT CGC CAC TG	2
qnrB-R	TTY GCB GYY CGC CAG TCG	1
qnrS-F	GCA AGT TCA TTG AAC AGG GT	2
qnrS-R	TCT AAA CCG TCG AGT TCG GCG	2
qepA-F	CGT GTT GCT GGA GTT CTT C	3
qepA-R	CTG CAG GTA CTG CGT CAT G	3
tetA-F	GCT ACA TCC TGC TTG CCT TC	4
tetA-R	GCA TAG ATC GCC GTG AAG AG	4
tetB-F	TCA TTG CCG ATA CCA CCT CAG	4
tetB-R	CCA ACC ATC ATG CTA TTC CAT CC	4
tetC-F	CTG CTC GCT TCG CTA CTT G	4
tetC-R	GCC TAC AAT CCA TGC CAA CC	4
tetD-F	TGT GCT GTG GAT GTT GTA TCT C	4
tetD-R	CAG TGC CGT GCC AAT CAG	4
tetE-F	ATG AAC CGC ACT GTG ATG ATG	4
tetE-R	ACC GAC CAT TAC GCC ATC C	4
tetG-F	GCG CTN TAT GCG TTG ATG CA	5
tetG-R	ATG CCA ACA CCC CCG GCG	5
tetM-F	GTG GAC AAA GGT ACA ACG AG	5
tetM-R	CGG TAA AGT TCG TCA CAC AC	5
AAC(6')-Ib-F	TTG CGA TGC TCT ATG AGT GGC TA	6
AAC(6')-Ib-R	CTC GAA TGC CTG GCG TGT TT	6
intI1F	CCT CCC GCA CGA TGA TC	7
intI1R	TCC ACG CAT CGT CAG GC	7
intI2F	TTA TTG CTG GGA TTA GGC	8
intI2R	ACG GCT ACC CTC TGT TAT C	8
intI3F	AGT GGG TGG CGA ATG AGT G	8
intI3R	TGT TCT TGT ATC GGC AGG TG	8
5'-CS	GGC ATC CAA GCA GCA AG	9
3'-CS	AAG CAG ACT TGA CCT GA	9
qacE-F	ATC GCA ATA GTT GGC GAA GT	10

qacE-R	CAA GCT TTT GCC CAT GAA GC	10
sulR1	AAA AAT CCC ATC CCC GGR TC	11
orf513_6F	ATG GTT TCA TGC GGG TT	12
orf513_7R	CTG AGG GTG TGA GCG AG	12
RH506 (tniR)	TTC AGC CGC ATA AAT GGA G	13
aadA1R	GCC ACG GAA TGA TGT CGT CG	14
gyrA-F	AAA TCT GCC CGT GTC GTT GGT	15
gyrA-R	GCC ATA CCT ACG GCG ATA CC	15
parC-F	CTG AAT GCC AGC GCC AAA TT	15
parC-R	GCG AAC GAT TTC GGA TCG TC	15
CTX-F	SCV ATG TGC AGY ACC AGT AA	16
CTX-R	GCT GCC GGT YTT ATC VCC	16
B/O-F	GCG GTC CGG AAA GCC AGA AAA C	17
B/O-R	TCT GCG TTC CGC CAA GTT CGA	17
FIC-F	GTG AAC TGG CAG ATG AGG AAG G	17
FIC-R	TTC TCC TCG TCG CCA AAC TAG AT	17
A/C-F	GAG AAC CAA AGA CAA AGA CCT GGA	17
A/C-R	ACG ACA AAC CTG AAT TGC CTC CTT	17
P-F	CTA TGG CCC TGC AAA CGC GCC AGA AA	17
P-R	TCA CGC GCC AGG GCG CAG CC	17
T-F	TTG GCC TGT TTG TGC CTA AAC CAT	17
T-R	CGT TGA TTA CAC TTA GCT TTG GAC	17
K/B-F	GCG GTC CGG AAA GCC AGA AAA C	17
K/B-R	TCT TTC ACG AGC CCG CCA AA	17
W-F	CCT AAG AAC AAC AAA GCC CCC G	17
W-R	GGT GCG CGG CAT AGA ACC GT	17
FIIA-F	CTG TCG TAA GCT GAT GGC	17
FIIA-R	CTC TGC CAC AAA CTT CAG C	17
FIA-F	CCA TGC TGG TTC TAG AGA AGG TG	17
FIA-R	GTA TAT CCT TAC TGG CTT CCG CAG	17
FIB-F	GGA GTT CTG ACA CAC GAT TTT CTG	17
FIB-R	CTC CCG TCG CTT CAG GGC ATT	17
Y-F	AAT TCA AAC AAC ACT GTG CAG CCT G	17
Y-R	GCG AGA ATG GAC GAT TAC AAA ACT TT	17
I1F	CGA AAG CCG GAC GGC AGA A	17
I1-R	TCG TCG TTC CGC CAA GTT CGT	17
X-F	AAC CTT AGA GGC TAT TTA AGT TGC TGA T	17

X-R	TGA GAG TCA ATT TTT ATC TCA TGT TTT AGC	17
HI1-F	GGA GCG ATG GAT TAC TTC AGT AC	17
HI1-R	TGC CGT TTC ACC TCG TGA GTA	17
N-F	GTC TAA CGA GCT TAC CGA AG	17
N-R	GTT TCA ACT CTG CCA AGT TC	17
HI2-F	TTT CTC CTG AGT CAC CTG TTA ACA C	17
HI2-R	GGC TCA CTA CCG TTG TCA TCC T	17
L/M-F	GGA TGA AAA CTA TCA GCA TCT GAA G	17
L/M-R	CTG CAG GGG CGA TTC TTT AGG	17
Frep-F	TGA TCG TTT AAG GAA TTT TG	17
Frep-R	GAA GAT CAG TCA CAC CAT CC	17
ERIC1R	ATG TAA GCT CCT GGG GAT TCA C	18
ERIC2	AAG TAA GTG ACT GGG GTG AGC G	18
qnrVC_F	GGA TAA AAC AGA CCA GTT ATA TGT ACA AG	This study*
qnrVC_R	AGA TTT GCG CCA ATC CAT CTA TT	This study

* PCR reaction mixtures (25 µl) had the following composition: 1× PCR buffer (PCR buffer with (NH4)2SO4), 3 mM MgCl2, 5% dimethylsulfoxide, 100 µM each nucleotide, 7.5 pmol of each primer, 0.5 U of Taq polymerase, and 50–100 ng of purified DNA. The temperature profile was as follows: initial denaturation (94ºC for 9 min); 30 cycles of denaturation (94ºC for 30 s), annealing (48ºC for 30 s), and extension (72ºC for 1 min); and a final extension (72ºC for 10 min);

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Part II

RESISTANCE TO CARBAPENEMS IN NATURAL ENVIRONMENTS

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3.3

RESISTANCE TO CARBAPENEMS IN RIVER WATER BACTERIA: POLLUTED *VS*. UNPOLLUTED ENVIRONMENTS

Abstract

Carbapenems are last-resort antibiotics for handling infections caused by multiresistant bacteria. The incidence of resistance to these antibiotics has been increasing and new resistance mechanisms have emerged. Despite its public health relevance, the dissemination of carbapenems resistance in the environment has been overlooked. The main goals of this research were to assess the prevalence and diversity of carbapenem-resistant bacteria in polluted and unpolluted rivers and to study the diversity of carbapenemase genes. The study was conducted in 11 rivers in Portugal. Imipenem-resistant bacteria incidence was higher in polluted rivers. Imipenem-resistant strains (n=110) were identified as *Pseudomonas* spp., followed by *Stenotrophomonas maltophilia*, *Aeromonas* spp., *Chromobacterium haemolyticum*, *Shewanella xiamenensis* and Enterobacteriaceae members, with no clear differences between polluted and unpolluted rivers in terms of phylogenetic diversity. High levels of beta-lactams resistance were observed in both environments with slightly higher numbers of strains resistant to quinolones, aminoglycosides, chloramphenicol and sulfamethoxazole/trimethoprim in polluted rivers. Multiresistance was observed in 70% of strains, and resistance to all antibiotic classes tested (6 classes) was higher among isolates from polluted sources. The $bla_{\text{VIM-2}}$ was detected in 5.45% of strains, all isolated from polluted rivers. Integrons were identified in *Pseudomonas* spp., with gene cassettes encoding resistance to aminoglycosides (*aac*A and *aac*C genes), trimethoprim (*dfr*B1b) and beta-lactams ($bla_{\text{VIM-2}}$). Carbapenems resistance was mostly associated with intrinsically-resistant bacteria. Nevertheless results show that resistance to carbapenems is being enhanced by anthropogenic pressures. As carbapenems resistance is still at an early stage, it is important to carry on monitoring these environments, to identify the dissemination promoters and so outline strategies to minimize this process.

3.3.1. INTRODUCTION

Over the years, the extensive use of antibiotics beyond medical practices has increased not only the prevalence of antibiotic-resistant bacteria and antibiotic-resistance genes but also the load of antibiotics discharged in the environment (Baquero *et al.* 2008, Lupo *et al.* 2012, Martinez 2009). Aquatic systems are the main collectors of antibiotics, mostly still in an active form, as well as of other compounds human-originated (e.g. disinfectants, metals) that accumulate and persist throughout time (Baquero *et al.* 2008, Lupo *et al.* 2012, Martinez 2009, Martinez 2009a, Taylor *et al.* 2011). Although antibiotics might remain in low concentrations, their presence, along with these other compounds, impose important selective pressures (Andersson and Hughes 2012). Adding the fact that there are antibiotic producers and/or bacteria that are intrinsically resistant to several antibiotics, these environmental reservoirs facilitate the spread of multidrug resistance features to human pathogenic bacteria (Lupo *et al.* 2012, Taylor *et al.* 2011). It has already been shown that anthropogenic pressures promote antibiotic resistance spread in the environment and that mobile genetic elements play an important role on these events (Tacão *et al.*, 2012; Tacão *et al.*, 2014; Taylor *et al.* 2011). Hence, aquatic systems must not be neglected when evaluating antibiotic resistance dispersion.

Carbapenems such as imipenem, meropenem and ertapenem are considered last-resort antibiotics, commonly applied to treat severe infections when all other therapeutic options fail (Bush 2013). In some countries, including Portugal, carbapenems use is still limited to hospital settings (Henriques *et al.* 2012). However, in the latter years the prevalence of bacterial resistance to carbapenems has continuously been increasing and while some resistance mechanisms are still geographically constrained, others are spread worldwide (Patel and Bonomo 2013). The most common carbapenem resistance mechanism reported in Gram-negative bacteria is the production of carbapenemases.

Clinically-relevant carbapenems-hydrolyzing beta-lactamases have been detected mostly in Enterobacteriaceae but there are also reports of carbapenemase production in other clinically important genera such as *Pseudomonas* and *Acinetobacter* (Bush 2013). Carbapenemases belong to 3 of the Ambler classes: class A (e.g. KPC), class B (e.g. VIM, NDM) and the class D (e.g. OXA-48) (Bush 2013).

Despite the fact that environmental resistance dissemination has been recognized as a major public health problem, the study of carbapenem resistance dissemination and the diversity of carbapenemases-encoding genes in the environment has been overlooked. Even so, carbapenemases have been described in environmental isolates, and in fact some have been detected only in environmental strains as for example BIC-1 in *Pseudomonas fluorescens* (Girlich *et al.* 2010),and Sfh-I and SFC-1 in *Serratia fonticola* (Henriques *et al.* 2004, Saavedra *et al.* 2003). On the other hand, clinically-relevant carbapenemases have also been identified in strains isolated from different environmental sources, such as KPC (Chagas *et al.* 2011, Picão *et al.* 2013, Poirel *et al.* 2012), VIM (Chouchani *et al.* 2013, Quinteira *et al.* 2005, Quinteira *et al.* 2006), IMP (Chouchani *et al.* 2013) and NDM (Isozumi *et al.* 2012, Walsh *et al.* 2011, Zhang *et al.* 2013) detected in strains isolated from rivers and/or waste waters. Moreover, for several carbapenems-hydrolyzing beta-lactamases the putative origin has been acknowledged to species that are commonly found in natural settings. Two examples are the class D carbapenemases OXA-23 in *Acinetobacter radioreducens* (Poirel *et al.* 2008) and OXA-48 in *Shewanella* spp. (Poirel *et al.* 2004, Tacão *et al.* 2013).

The majority of these observations resulted from large screening investigations where a few carbapenem-resistant strains were identified. Few studies have focused specifically in the study of bacterial resistance towards these last-resort antibiotics in natural environments (Henriques *et al.* 2012) and so data on the diversity of carbapenem-resistant bacteria and their resistance mechanisms in these settings is still scarce.

The main goals of this research were to assess the prevalence and diversity of carbapenem-resistant bacteria in polluted and unpolluted rivers and to study the diversity of carbapenemase encoding genes.

3.3.2 MATERIAL AND METHODS

3.3.2.1 Sample collection

Water samples were collected in 12 locations in 11 rivers in the Vouga River basin, located in central Portugal. These sampling sites are impacted by different pollution sources, of agricultural, industrial and domestic origins. Previously analyzed physical, chemical and microbiological parameters showed that these rivers displayed different levels of superficial water quality, from unpolluted to polluted characteristics: 3 rivers were classified as polluted and 9 as unpolluted, according to the national legislation for water quality categorization (for details see Tacão *et al.* 2012). Water was collected in sterile bottles (7L) from 50 cm below the water surface and kept on ice for transportation.

3.3.2.2 Enumeration and selection of imipenem-resistant bacteria

Water samples were filtered in sterile 0.45-µm-pore-size cellulose ester filters, and the membranes placed on MacConkey agar plates supplemented with 8 µg/ml of imipenem. MacConkey medium was used to select for Gram-negative phylogenetic groups that are currently the greatest threats in terms of carbapenemase resistance (Nordmann *et al.*, 2011; Bush, 2013). The total filtered volumes varied from 1 mL to 500 mL, according to preliminary studies conducted in each sampling site. Plates without an antibiotic supplement were used to determine the proportion of imipenem-resistant bacteria. Plates were incubated at 37ºC for 16 h. Colony counting was done in triplicate. Individual imipenem-resistant colonies were purified and stored in 20% glycerol at −80ºC.

3.3.2.3 Molecular typing and identification of imipenem-resistant isolates

BOX-PCR was used to type all isolates as previously described (Tacão *et al.*, 2012). PCR products were loaded in 1.5% agarose gels for electrophoresis and banding patterns were analyzed with the software GelCompar II version 6.1 (Applied Maths, Belgium, available from http://www.applied-maths.com/). Similarity matrices were calculated with the Dice coefficient and cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages (UPGMA; Sneath and Sokal, 1973). Isolates displaying different BOX profiles were identified by 16S rRNA gene sequencing analysis with primers and PCR conditions as previously described (Tacão *et al.*, 2012). PCR products were purified with DNA Clean & Concentrator (Zymo Research, USA) following manufacturer's instructions, and used as template in the sequencing reactions. Online similarity searches were performed with the BLAST software at the National Center for Biotechnology Information website against the GenBank database. Identification was confirmed with the EZTaxon tool available at http://www.ezbiocloud.net/eztaxon#, using on average 1200 bp.

3.3.2.4 Antibiotic susceptibility testing

Antibiotic susceptibility patterns were determined by the agar disc diffusion method on Mueller–Hinton agar, against 14 antibiotics from 6 classes: beta-lactams (penicillins, monobactams, carbapenems, beta-lactam/beta-lactamase combination and 3rd and 4th generation cephalosporins), quinolones, aminoglycosides, phenicols, tetracyclines and the combination sulfamethoxazole/trimethoprim. After 24 h of incubation at 37ºC, results were analyzed following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST 2014). In the lack of EUCAST information, the Clinical Laboratory Standards Institute criteria were used (CLSI 2012). Detection of extended-spectrum beta-lactamase (ESBL) production was carried out by a clavulanic acid combination disc method based on comparing the inhibition zones of cefpodoxime (10 μg) and cefpodoxime-plus-clavulanate (10/1 μg) discs (Oxoid, UK).

3.3.2.5 PCR amplification of antibiotic resistance determinants

Genes conferring resistance to beta-lactams (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{SPM}$, *bla*$_{AIM}$, *bla*$_{GIM}$, *bla*$_{DIM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{KPC}$, *bla*$_{GES}$, *bla*$_{NDM}$, *bla*$_{CphA-like}$, *bla*$_{L1}$, *bla*$_{CTX-M}$, *bla*$_{PER}$, *bla*$_{VEB}$), to tetracycline [*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(O) and *tet*(M)] and fluoroquinolones (*qnrA*, *qnrB*, *qnrVC*, and *aacA4-cr*) were inspected by PCR using previously described primers and conditions (see Table 1). Results were confirmed by sequencing.

TABLE 1: Primers used in this study.

Resistance to	**Primers targeting genes**	**References**
Beta-lactams	*bla*$_{SHV}$	Henriques *et al.* 2006
	bla$_{TEM}$	Speldooren *et al.* 2006
	bla$_{GES}$, *bla*$_{VEB}$, *bla*$_{PER}$, *bla*$_{KPC}$	Dallenne *et al.* 2010
	bla$_{IMP}$, *bla*$_{VIM}$	Henriques *et al.* 2006
	bla$_{AIM}$, *bla*$_{SPM}$, *bla*$_{GIM}$, *bla*$_{DIM}$, *bla*$_{NDM}$	Poirel *et al.* 2011
	bla$_{CTX-M}$	Lu *et al.* 2010
	bla$_{L1}$	Avison *et al.* 2001
	bla$_{CphA-like}$	Henriques *et al.* 2006
Quinolones	*qnr*A, *qnr*B	Cattoir *et al.* 2007 Guillard *et al.* 2011
	*qnr*VC	Tacão *et al.* 2014
	aacA4-cr	Park *et al.* 2006
Tetracyclines	*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E)	Nawaz *et al.* 2006
	tet(G), *tet*(M), *tet*(O)	Ng *et al.* 2001

3.3.2.6 Integron screening and characterization

Integrase screening was performed for *intI*1 and *intI*2 genes (Henriques *et al.* 2006). The variable regions of integrase-positive strains were further amplified by PCR as described before (Tacão *et al.* 2014) and sequenced.

3.3.2.7 Statistical analysis

Statistical analysis was performed by two-sample t-test using GraphPad Prism for Windows (GraphPad Software, San Diego, CA, USA).To assess correlations between antimicrobial susceptibility profiles and the isolation source (polluted *vs*. unpolluted), a cluster analysis was performed. For that results were converted into a binary matrix (1, resistant to the antibiotic; 0, susceptible to the antibiotic). Similarity matrices were calculated using the Bray-Curtis coefficient and cluster analysis was performed using UPGMA. The analysis was performed with the PRIMER 6 software (Clarke 2006).

3.3.2.8 Nucleotide sequence accession numbers

All the nucleotide sequences stated in this work have been deposited in the GenBank database under the accession numbers KJ396795–KJ396890 (16S rRNA gene sequences), KJ620481 - KJ620486 (bla_{VIM} genes from bacterial isolates), KM495226 - KM495239 (bla_{cphA} genes from bacterial isolates) and KM495240 - KM495266 (bla_{L1} genes from bacterial isolates)

3.3.3 RESULTS

3.3.3.1 Prevalence and phylogenetic diversity of imipenem-resistant bacteria

Bacterial counts on MacConkey agar were on average 10^5 CFU/100mL of riverine water of which 0.19% grew on MacConkey agar supplemented with imipenem ($1.87X10^2$ CFU/100mL). Comparing bacterial counts in polluted and unpolluted rivers, higher prevalence of imipenem-resistant bacteria was observed in polluted rivers, with 0.34% vs. 0.03% in unpolluted rivers, although not statistically significant. Among polluted rivers, higher numbers (statistically significant, $p< 0.05$) were observed in the water of the only river that was classified as extremely polluted, showing high values

for several water quality parameters previously determined (Tacão *et al.*, 2012), which include high phosphates and ammonia concentrations (2.7 mg/L and 5.5 mg/L, respectively), high load of fecal streptococci, total and fecal coliforms (all above 500 CFUs/100 mL). The prevalence of carbapenem-resistant bacteria in this river was the highest observed in this study, with 20.5 UFCs/mL.

Clonal relationships among imipenem-resistant isolates (n=184) were evaluated by BOX-PCR, and 110 isolates displaying unique BOX profiles were selected for sequencing analysis of 16S rRNA gene. Identification results are shown in Table 2.

Overall, the most frequent genus with 41.8% of the total number of strains, was *Pseudomonas* (*P. geniculata*, *P. beteli*, *P. hibiscicola*, *P. aeruginosa*, *P. monteilli*, *P. protegens*, *P. otitidis*, *P. putida*, *P. taiwanensis* and *Pseudomonas* sp.), followed by *Stenotrophomonas maltophilia* with 24.5%, *Aeromonas* adding 20% (*A. veronii*, *A. hydrophila*, *A. jandaei*, *A. australlensis*), *Chromobacterium haemolyticum* with 8.2% and finally both with 2.7%, *Shewanella xiamenensis* and Enterobacteriaceae members (*Enterobacter ludwigii*, *Enterobacter asburiae* and *Providencia alcaligenes*). There were no relevant differences between polluted and unpolluted rivers in terms of the phylogenetic distribution of the retrieved carbapenem-resistant strains (Table 2).

3.3.3.2 Antimicrobial susceptibility testing

Levels of resistance of isolates from polluted and unpolluted rivers are shown in figure 1. Overall, imipenem-resistant strains showed resistance to ampicillin and to both carbapenems tested (imipenem and ertapenem). Also 88.2% of total strains showed resistance to the 3rd generation cephalosporin cefotaxime and 62.7% to the 4th generation cephalosporin cefepime. For non-beta-lactam antibiotics, higher resistance levels were observed against aminoglycosides (particularly against kanamycin with 68.2% resistant isolates), followed by resistance towards tetracycline (50.9%), nalidixic acid (43.6%), chloramphenicol (43.6%) and sulfamethoxazole/trimethoprim (33.6%). No ESBL was detected by the double disc diffusion test.

TABLE 2: Phylogenetic affiliation and distribution of bacterial strains among polluted and unpolluted rivers.

	POLLUTED			**UNPOLLUTED**		
Identification	**Nº of isolates**	**Incidence**		**Nº of isolates**	**Incidence**	
Pseudomonas sp.	4	9.3%	37.2% (n=16)	0	-	44.8% (n=30)
Pseudomonas geniculata	3	6.9%		11	16.4%	
Pseudomonas beteli	0	-		5	7.5%	
Pseudomonas hibiscicola	1	2.3%		2	3.0%	
Pseudomonas aeruginosa	3	6.9%		1	1.5%	
Pseudomonas protegens	2	4.7%		7	10.4%	
Pseudomonas otitidis	1	2.3%		4	5.9%	
Pseudomonas putida	1	2.3%		0	-	
Pseudomonas taiwanensis	1	2.3%		0	-	
Stenotrophomonas matophilia	12	27.9%		15	22.4%	
Aeromonas veronii	5	11.6%	23.3% (n=10)	1	1.5%	17.9% (n=12)
Aeromonas jandaei	0	-		3	4.5%	
Aeromonas australlensis	0	-		1	1.5%	
Aeromonas hydrophila	5	11.6%		7	10.4%	
Chromobacterium haemolyticum	1	2.3%		8	11.9%	
Shewanella xiamenensis	2	4.7%		1	1.5%	
Enterobacter ludwigii	1	2.3%	4.6% (n=2)	0	-	1.5% (n=1)
Enterobacter asburiae	1	2.3%		0	-	
Providencia alcaligenes	0	-		1	1.5%	

As stated above, high levels of antibiotic resistance were observed towards beta-lactams in both environments (FIG. 1), and so differences between polluted and unpolluted settings were only noticed for resistance to some non-beta-lactam antibiotics.

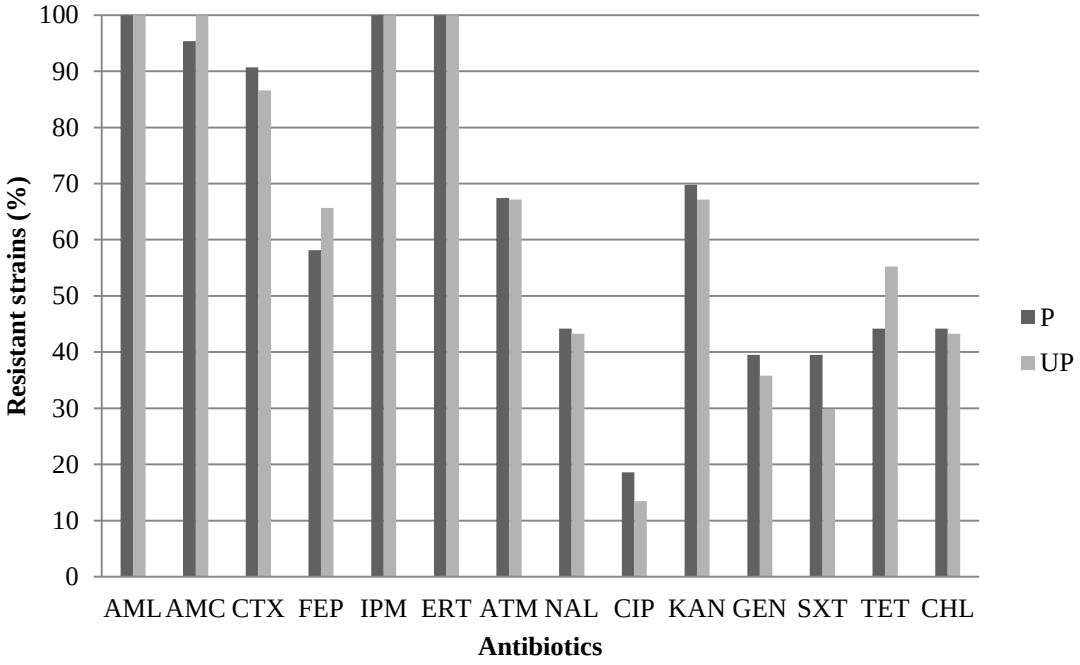


FIG. 1: Prevalence of strains (%) in polluted (P, dark grey) and unpolluted (UP, light grey) river water resistant to: AML-amoxicillin, AMC- Amoxicillin + clavulanic acid, CTX- cefotaxime, FEP- cefepime, IPM- imipenem, ERT- ertapenem, ATM- aztreonam, NAL- nalidixic acid, CIP- ciprofloxacin, KAN- kanamycin, GEN- gentamicin, SXT- sulfamethoxazole-thrimetoprim, TET- tetracycline, CHL- chloramphenicol.

Resistance towards quinolones (nalidixic acid and ciprofloxacin), aminoglycosides (kanamycin and gentamicin), chloramphenicol and sulfamethoxazole/trimethoprim was slightly higher among strains isolated from polluted river water, with *Pseudomonas* strains contributing the most for these observations (FIG. 2).

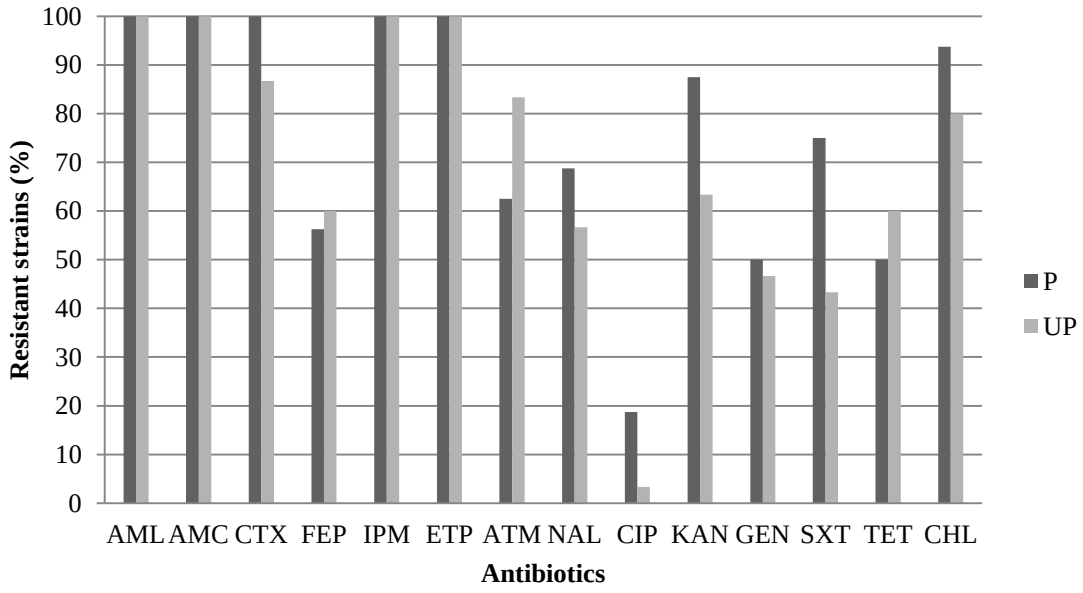


FIG. 2: Prevalence of *Pseudomonas* strains (%) in polluted (P, dark grey) and unpolluted (UP, light grey) river water resistant to: AML-amoxicillin, AMC- Amoxicillin + clavulanic acid, CTX- cefotaxime, FEP- cefepime, IPM- imipenem, ETP- ertapenem, ATM- aztreonam, NAL- nalidixic acid, CIP- ciprofloxacin, KAN- kanamycin, GEN- gentamicin, SXT- sulfamethoxazole-thrimetoprim, TET- tetracycline, CHL- chloramphenicol.

In fact, resistance levels observed among pseudomonads showed clear differences between strains isolated from polluted and those from unpolluted river water, particularly towards sulfamethoxazole/trimethoprim, ciprofloxacin and kanamycin.

Multiresistance (defined as resistance to 3 or more classes of antibiotics, including beta-lactams) was found in 70% of the strains (n=77). Overall, *Pseudomonas* spp. contributed the most, representing 57.1% of the multiresistant strains, followed by *S. maltophilia* strains with 28.6%. In fact, 95.6% and 84.5% of pseudomonads and *S. maltophilia* strains, respectively, were multiresistant (44 out of 46 *Pseudomonas* spp. and 22 out of 27 *S. maltophilia* strains). Aeromonads contributed with 8.2% of total multiresistance (40.9% of total *Aeromonas* strains; 9 out of 22). Two out of 3 Enterobacteriaceae strains were multiresistant. The prevalence of strains resistant towards all antibiotic classes tested (6 classes) was higher among isolates from polluted than those from unpolluted waters (FIG. 3). Multiresistance phenotypes were not identified in neither *C. haemolyticum* nor *S. xiamenensis* strains.

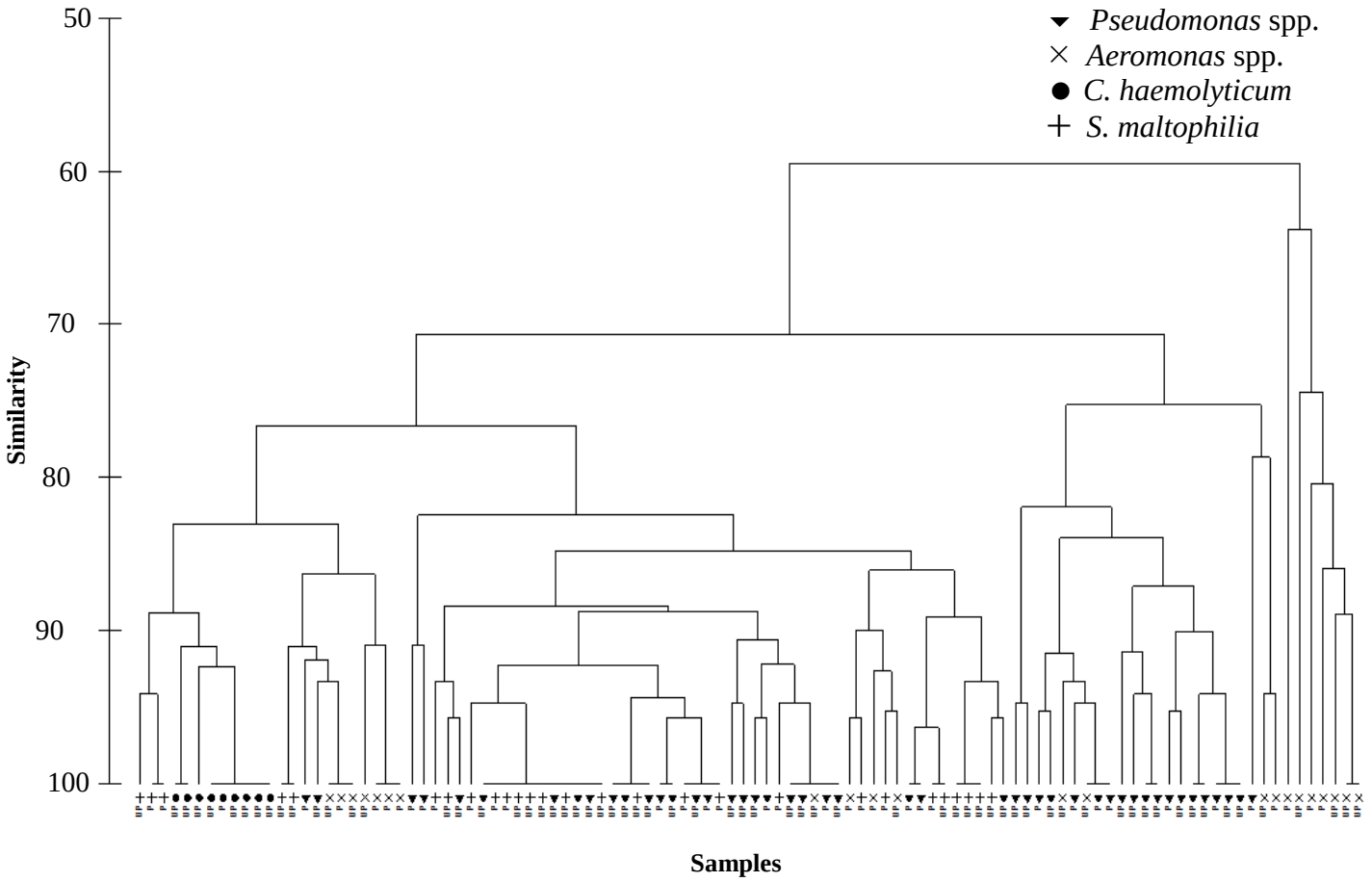
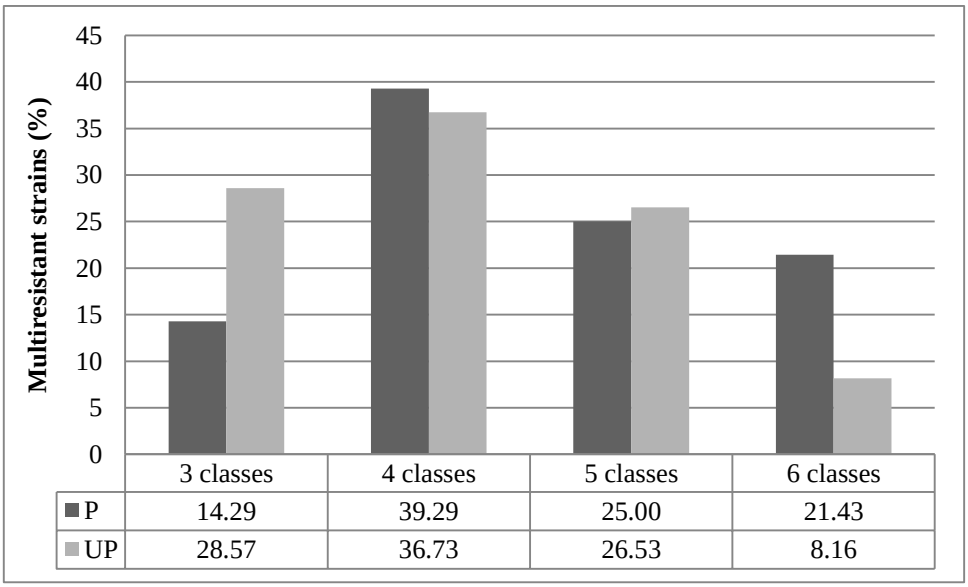


FIG.3: Clustal analysis of the antibiotic susceptibility profiles of *Pseudomonas*, *Aeromonas*, *S. maltophilia* and *C. haemolyticum* strains isolated from polluted (P) and unpolluted (UP) river water, using Bray-Curtis similarity coefficient and UPGMA cluster methods.

By comparing polluted *vs* unpolluted environments by cluster analysis of all the antibiotic susceptibility profiles (FIG. 4) we observed that strains group preferentially according to their phylogenetic affiliation rather than water quality (i.e. polluted and unpolluted).



	3 classes	4 classes	5 classes	6 classes
P	14.29	39.29	25.00	21.43
UP	28.57	36.73	26.53	8.16

FIG. 4: Prevalence of multiresistant strains (%) in polluted (P, dark grey) and unpolluted (UP, light grey) river water resistant to 3 up to 6 classes of antibiotics.

3.3.3.3 Occurrence and diversity of antibiotic resistance genes

The imipenem-resistant isolates were further analyzed by PCR with the primer sets specific to the antibiotic resistance genes. The carbapenemase genes $bla_{\text{CphA-like}}$ were detected in 77.3% of *Aeromonas* spp. (17 out of 22) and the bla_{L1} gene in all *S. maltophilia* (n=27). bla_{VIM} was detected in 6 *Pseudomonas* strains isolated from polluted waters.

Sequencing results showed that the 6 $bla_{\text{VIM-positive}}$ strains (1 *P. putida*, 1 *P.monteilii*, 1 *P. geniculata* and 2 *Pseudomonas* sp.) carried a $bla_{\text{VIM-2}}$ gene.

Genes conferring resistance to tetracyclines, aminoglycosides or quinolones were not detected with the primers used in this study.

3.3.3.4 Integrons characterization

The gene *intI*1 was detected in 2 *Pseudomonas* strains (IR35 and IR49) isolated from polluted waters, both carrying the carbapenemase gene $bla_{\text{VIM-2}}$. The integrons variable regions were analyzed. The gene cassette arrays identified conferred resistance

to aminoglycosides (*aac*A and *aac*C type genes), trimethoprim (*dfr*B1b) and beta-lactams (bla_{VIM-2}). In *P. putida* IR35 the gene cassette array was *aac*A7-bla_{VIM-2}-*aac*C1-*aac*A4 and in *P. geniculata* IR49 was *dfr*B1b-*aac*A4-bla_{VIM-2}. The gene cassette array bla_{VIM-2}–*aac*A4 was identified in 4 *Pseudomonas* spp. strains (IR46, IR52, IR53 and IR54) using primers targeting both genes, but no integrase gene was detected.

3.3.4 DISCUSSION

No doubt that the increasing number of antibiotic-resistant bacterial strains is a serious public health concern that has been addressed in many studies worldwide. Particularly worrying are the growing numbers of clinically-relevant strains resistant to last-resort antibiotics such as carbapenems. Despite the clear public health importance, few studies have addressed this topic.

Here, we focused on the riverine carbapenems resistome, in what concerns prevalence and diversity of carbapenem-resistant bacteria, resistance genes and mechanisms of resistance dissemination, in polluted and unpolluted aquatic environments.

The prevalence of imipenem-resistant bacteria was low. In comparison with the incidence of cefotaxime-resistant bacteria (Tacão *et al.* 2012) calculated for the same rivers and sampling period, the proportion of imipenem-resistant isolates was clearly inferior (0.18% *vs.* 4.64%, on average). In a previous study performed in Portugal with bacteria from untreated drinking water, results showed also a low prevalence of imipenem-resistant bacteria (Henriques *et al.* 2012). These numbers might be linked to carbapenems restrictive administration in Portuguese clinics (Henriques *et al.* 2012). The lack of similar studies in different geographic regions, with more permissive carbapenems prescription policies, prevents a comparison that would be of major interest.

Noteworthy, in this study, a very high prevalence of imipenem-resistant bacteria (> 20%) was detected in the river classified as extremely polluted, indicating that, as observed previously for cefotaxime-resistant bacteria (Tacão *et al.* 2012), anthropogenic activities might influence the prevalence of carbapenems resistance in these aquatic

systems. This river is impacted by different sources of pollution which include not only those of domestic and agricultural origins but also industry related sources (Tacão *et al.*, 2012). The high levels of imipenem resistance detected in this river might be related to co-selection events driven by the presence of other contaminants rather than antibiotics. Similar effects were reported in other studies (Baker-Austin *et al.* 2006, Seiler and Berendonk 2012).

As expected, since bacteria were isolated in imipenem-containing culture media, high resistance rates were observed towards beta-lactams. Most commonly, cross-resistance mechanisms, that is, the same resistance determinant conferring resistance to more than one antibiotic, are responsible for this extended phenotype. For example metallo-beta-lactamases like VIM or IMP are able to hydrolyze all beta-lactams (Pfeifer *et al.* 2010).

The isolation of carbapenem-resistant *Aeromonas* and *S. maltophilia* was of no surprise. These are commonly isolated from aquatic systems and intrinsically resistant to carbapenems (Lupo *et al.*2012, Patel and Bonomo 2013). In *S. maltophilia* resistance results from the expression of bla_{L1}, encoded in a plasmid-like element considered intrinsic to this species (Avison *et al.* 2001). bla_{L1} was detected in all *S. maltophilia* strains isolated in this study. Likewise, the majority of members of the genus *Aeromonas* show resistance towards carbapenems due to the expression of chromosomal class B metallo-beta-lactamase genes like bla_{CphA} (Walsh *et al.* 2005), which was detected in the majority of aeromonads here isolated.

The majority of imipenem-resistant strains isolated belonged to the genus *Pseudomonas*. Although in general carbapenems (except for ertapenem) are active against pseudomonads, several carbapenems resistance mechanisms have been described particularly in *P. aeruginosa*, which is by far the most studied species in this genus due to its clinical importance. Pseudomonads might carry one or combinations of 2 or more resistance mechanisms which include high-level expression of chromosomally encoded class C β-lactamase, reduced outer membrane permeability and overexpression of efflux pumps with wide substrate specificity (Livermore 2001, Mesaros *et al.* 2007, Strateva and Yordanov 2009). These combinations act differently according to the antibiotic molecule (Strateva and Yordanov 2009). Also plasmid-mediated class A (e.g. BIC-1, GES- and KPC-types) and class B (e.g. IMP- and VIM-

types) carbapenemases (Patel and Bonomo 2013) have been detected in *Pseudomonas* strains. Recently, a 3-year surveillance study performed with *P. aeruginosa* isolates obtained in hospitals in 14 European countries, showed that, although there was an increase in the number of metallo-beta-lactamase producers (most frequently VIM-2), the majority carried 1 or more resistance mechanisms, being the loss of OprD (reduced permeability) the most common cause for the high minimal inhibitory concentration (MIC) values observed (Castanheira *et al.* 2014). Moreover, combinations of these intrinsic mechanisms have been associated to resistance to unrelated classes of antibiotics, which might result in resistance to all beta-lactams but also quinolones and aminoglycosides (Strateva and Yordanov 2009). Also for the majority of pseudomonads analyzed in this study MIC values for ertapenem, meropenem and imipenem were over 32 µg/mL (data not shown).

In general, only slight differences were observed between the antibiotic susceptibility profiles of strains retrieved from polluted and unpolluted river water, yet, when analyzed separately, *Pseudomonas* spp. isolated from polluted settings presented higher resistance levels particularly towards non beta-lactams. Although globally multiresistance levels were high, in fact resistance towards 6 classes of antibiotics was almost 3 times higher among strains isolated from polluted water. These results show evidences that water quality is determining antibiotic resistance levels, i.e., anthropogenic pressures are modulating the carbapenems resistome in these aquatic environments.

More than half of strains presenting multiresistance phenotype affiliated with *Pseudomonas*. The majority of these strains presented resistance to several classes of non-beta-lactam antibiotics, mostly towards aminoglycosides, quinolones and chloramphenicol. In *Pseudomonas* spp. several resistance determinants have been described previously encoding resistance to these antibiotics (Tacão *et al.* 2014, van Hoek *et al.* 2011). Although we have targeted a large number of these genes none was detected by PCR with the primers used in this study, and most probably, intrinsic resistance mechanisms are responsible for the phenotypes observed.

We have detected $bla_{\text{VIM-2}}$ genes 100% identical to those reported in clinics. This is the most common VIM variant reported so far in clinical settings worldwide (Patel and Bonomo 2013), including Portugal although sporadically (Nordmann *et al.* 2011,

Cantón *et al.* 2012). The *bla*$_{\text{VIM-2}}$ was identified as gene cassette included in arrays with other resistance genes, as frequently described (Patel and Bonomo 2013). Hence, with this multiresistance apparatus several classes of antibiotics are covered. Both gene cassette arrays have been already described in clinical *P. aeruginosa* strains (http://integrall.bio.ua.pt; Moura *et al.* 2009). Although the prevalence of these usually acquired genes is still low in these water bodies, their presence suggest that the dissemination of acquired carbapenems resistance is at an early stage. Their association to mobilizable genetic platforms simplifies their dispersion and the fact that *bla*$_{\text{VIM-2}}$ genes were detected only in strains isolated from polluted river water alert to the fact that anthropogenic pressures can haste these events.

3.3.5 CONCLUSIONS

The prevalence of carbapenem-resistant bacteria in aquatic environments is still low and mostly related to the presence of intrinsically resistant bacteria, at least in countries where carbapenems prescription policies are restrictive, as in Portugal. However we gathered evidences that show that the dissemination of carbapenems resistance might be accelerated by human-related pressures.

These findings warn for the relevance of monitoring anthropogenic activities, which include contaminants disposal in these environments, comprising not only antibiotics but also antibiotic resistance genes, antibiotic resistant bacteria, and other pollutants such as metals and disinfectants. These contaminants have been proven to promote also antibiotic resistance dissemination, with mobile genetic elements as main mediators.

Hence, since carbapenems resistance dissemination is apparently at its initial phase, it gives the opportunity to monitor these environments and to identify and minimize the key human-derived negative impacts that are reducing water quality continuously and consequently promoting resistance dissemination.

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3.4

ENVIRONMENTAL *Shewanella xiamenensis* STRAINS THAT CARRY *bla*$_{OXA-48}$ OR *bla*$_{OXA-204}$ GENES: ADDING PROOF FOR *bla*$_{OXA-48-like}$ GENES ORIGIN

The chromosome-encoded beta-lactamases of *Shewanella* spp. have been recognized as progenitors of *bla*$_{OXA-48-like}$ genes (Poirel *et al.* 2012). The analysis of available genome sequences of *Shewanella* spp. showed the presence of *bla*$_{OXA-48-like}$ genes in their chromosome with at least 80% identity to *bla*$_{OXA-48}$ (Zong 2012). Although initially considered as geographically restricted, it has now been demonstrated that the spread of the *bla*$_{OXA-48}$ gene is one of the greatest concerns in terms of antibiotic resistance (Patel and Bonomo 2013). In fact, since its first description less than a decade ago (Poirel *et al.* 2004), *bla*$_{OXA-48-like}$ genes have been reported worldwide (Poirel *et al.* 2012, Patel and Bonomo 2013). Several variants of *bla*$_{OXA-48}$ genes have been identified in Enterobacteriaceae strains, mostly isolated from clinical settings. So far, *bla*$_{OXA-181}$ (Potron *et al.* 2011), *bla*$_{OXA-48b}$ and *bla*$_{OXA-199}$ (Zong 2012) have been reported in *S. xiamenensis* strains.

The OXA-204 enzyme was recently described in *Klebsiella pneumoniae* clinical isolates in Tunisia. Its substrate profile is similar to OXA-48, from which differs by only two amino acids (Poirel *et al.* 2013). The origin of *bla*$_{OXA-204}$ was not identified before. Here we report the isolation of three *S. xiameniensis* strains from river water in Portugal, one of which carried the *bla*$_{OXA-204}$ gene. Strains IR24, IR33 and IR34 were isolated from rivers (Tacão *et al.* 2012) in MacConkey agar plates supplemented with 8 μg/ml of imipenem and identified by 16S-rDNA sequencing as *S. xiamenensis*. Sequencing of the *bla*$_{OXA-48-like}$ genes amplified by PCR using previously described primers (Zong 2012) revealed that these strains carried either a *bla*$_{OXA-48b}$ (IR24 and IR33) or a *bla*$_{OXA-204}$ gene (IR34).

Antimicrobial susceptibility and MICs were determined in Mueller-Hinton agar plates at 37ºC and interpreted according to the CLSI guidelines (CLSI 2012). Results are shown in table 1. All three isolates were resistant to penicillins and carbapenems but susceptible to 3rd generation cephalosporins and fluoroquinolones. MICs of ertapenem, imipenem and

meropenem for OXA-204-producing strain were at least 4 times higher than those determined for the OXA-48-producing strains. Moreover, MICs for carbapenems were also higher than those previously described for *K. pneumoniae* carrying $bla_{\text{OXA-204}}$ (6).

To investigate the genetic context, primers were designed targeting regions commonly described as flanking $bla_{\text{OXA-48-like}}$ genes in *Shewanella* spp. (Zong 2012): upstream a gene encoding peptidase C15 (C15_fwd: 5'- TTACGGCCTGGGAAGTGTTC-3') and downstream the *lysR* gene (lysR_rev: 5'- AAGGGATTCTCCCAAGCTGC-3') which codes for a putative LysR transcriptional regulator. Sequencing of the amplified region revealed an identical context for both $bla_{\text{OXA-204}}$ and the $bla_{\text{OXA-48}}$ genes, presenting upstream the C15 gene and downstream the *lysR* gene (accession numbers KC902850-KC902852). This constitutes the first report on *S. xiamenensis* strains carrying a $bla_{\text{OXA-204}}$ gene suggesting that the emergence of different $bla_{\text{OXA-48-like}}$ genes probably had origin in different *S. xiamenensis* strains. Also it suggests the participation of diverse mobilization events and mechanisms in the transfer of $bla_{\text{OXA-48-like}}$ genes from *Shewanella* spp. to Enterobacteriaceae. Whereas IS*Ecp1* has been identified preceding the $bla_{\text{OXA-204}}$ and $bla_{\text{OXA-181}}$ gene, the IS*1999* has been found upstream $bla_{\text{OXA-48}}$ genes (Poirel *et al.* 2012). Moreover, it is of great relevance to acknowledge that these genetic events may have occurred in natural environments, reinforcing the importance of aquatic systems on the evolution and spread of antibiotic resistance.

TABLE 1: Resistance phenotype and MICs of carbapenems for *S. xiamenensis* strains

Strain::*bla*OXA-48-like gene	Resistance phenotype	MIC (µg/ml)		
		ERT	IMP	MER
S. xiamenensis IR24::*bla*OXA-48	AML AMC IPM ERT ATM	8 (R)	4 (R)	2 (I)
S. xiamenensis IR33::*bla*OXA-48	AML AMC CTX IPM ERT ATM	8 (R)	4 (R)	1 (S)
S. xiamenensis IR34::*bla*OXA-204	AML AMC IPM ERT NA	>32 (R)	>32 (R)	8 (R)

AML- amoxicillin, AMC – amoxicillin + clavulanic acid, ATM- aztreonam, CTX- cefotaxime, ERT - ertapenem, IPM- imipenem, MER – meropenem, NAL- nalidixic acid

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3.5

CULTURE-INDEPENDENT METHODS REVEAL HIGH DIVERSITY OF OXA-48-LIKE GENES IN AQUATIC ENVIRONMENTS

Abstract

The carbapenemase OXA-48 was identified for the first time in 2001 and is now one of the greatest concerns in terms of antibiotic resistance. While many studies report clinical OXA-48-like producers, few reports refer blaOXA-48-like genes in environmental bacteria. The main goal of this study was to evaluate the diversity of blaOXA-48-like genes in aquatic systems, using culture-independent approaches. For that, environmental DNA was obtained from riverine and estuarine water and used to construct clone libraries of $bla_{\text{OXA-48-like}}$ gene PCR amplicons. $bla_{\text{OXA-48-like}}$ libraries from river and estuarine water DNA comprised 75 and 70 clones, respectively. Sequence analysis showed that environmental $bla_{\text{OXA-48-like}}$ genes span a broader diversity than that so far observed in clinical settings. In total, 50 new OXA-48 variants were identified as well as sequences identical to previously reported OXA-48, OXA-181, OXA-199, OXA-204 and OXA-162. These results reinforce that natural systems have been undervalued in what concerns antibiotic resistance-related investigations. Also strengthen the risk associated to natural reservoirs of $bla_{\text{OXA-48-like}}$ that persist and disseminate successfully, and that may pose a serious antibiotic resistance threat. The variants of $bla_{\text{OXA-48}}$ here described should be taken into account when designing molecular strategies for detecting this gene.

3.5.1 INTRODUCTION

The class D OXA carbapenemases comprises a diverse group of enzymes that have been identified mostly in outbreaks of carbapenem-resistant *Acinetobacter* spp. (e.g. OXA-23, OXA-24, OXA-40, and OXA-58), *Pseudomonas* spp. (OXA-50) and Enterobacteriaceae (OXA-48) (Evans and Amyes, 2014). Species-specific class D carbapenemases have also been identified like the OXA-60 family, naturally present in the genome of *Ralstonia pickettii* (Girlich *et al.* 2004), and OXA-62 in *Pandoraea pnomenusa* (Schneider *et al.* 2006).

The carbapenemase OXA-48 was identified for the first time in a clinical *Klebsiella pneumoniae* isolate in Turkey (Poirel *et al.* 2004). Although initially disseminated mostly in Mediterranean countries, nowadays OXA-48 and its variants are an example of widely disseminated carbapenemases that have been detected in all continents (Poirel *et al.* 2012). These enzymes hydrolyze penicillins and carbapenems, but not 3rd generation cephalosporins (Poirel *et al.* 2012). However, there are many reports of OXA-48-like-producers that carry also an extended spectrum beta-lactamase gene, commonly a $bla_{\text{CTX-M-15}}$ gene. The expression of both genes ($bla_{\text{OXA-48}}$ and $bla_{\text{CTX-M-15}}$) results in resistance to most beta-lactams, leading to limited treatment options (Poirel *et al.* 2012).

Since its first description $bla_{\text{OXA-48-like}}$ genes detection has been restricted to *Shewanella* species (Potron *et al.* 2011a, Poirel *et al.* 2012; Zong, 2012, Tacão *et al.* 2013) and Enterobacteriaceae members worldwide (Potron *et al.* 2011, Poirel *et al.* 2012; Galler *et al,* 2013, Gomez *et al.* 2013, Sampaio *et al.* 2014). Although most reports referred to clinical isolates, there are also reports describing OXA-48-producers in Enterobacteriaceae isolated from river water (Potron *et al.* 2011) and wastewater (Galler *et al* 2013).

Up to now, 11 OXA-48 variants have been found, differing in 1 to 5 amino acids: OXA-48, OXA-162, OXA-163, OXA-181, OXA-199, OXA-204, OXA-232, OXA-244, OXA-245, OXA-247 and OXA-370 (Poirel *et al.* 2012; Gomez *et al.* 2013, Sampaio *et al.* 2014).

Shewanella spp., the putative origin of OXA-48-like genes, are mostly identified in aquatic ecosystems, under a wide range of environmental conditions. Furthermore some members of this genus are increasingly being linked to cases of human infections, acquired mostly after exposure to water through professional- or leisure-related activities (Janda and Abbot, 2014). So far, the gene variants $bla_{\text{OXA-48}}$, $bla_{\text{OXA-199}}$ and $bla_{\text{OXA-204}}$ have been detected in shewanellae (Potron *et al.* 2011a, Zong, 2012; Tacão *et al.* 2013). Presumptively other *Shewanella* strains carrying diverse $bla_{\text{OXA-48-like}}$ genes are expected to be present in aquatic systems.

Although there have been reports on OXA-48-like carbapenemases worldwide, it has been pointed out that the spread of this beta-lactamase is silent due to the difficulties on its detection. In fact, OXA-48-producers show low Minimal Inhibitory Concentrations (MIC) values for carbapenems, which might be masking their presence leading to an underestimation of its dispersion (Poirel *et al.* 2012). Therefore, molecular methods have been pointed out as liable alternatives for the recognition of OXA-48-like producers (Poirel *et al.* 2012).

The study of the diversity of $bla_{\text{OXA-48-like}}$ genes is important for elaborating molecular-based strategies for their rapid detection. Also unrevealing the diversity of these molecular determinants can contribute to get insights into their evolution and to anticipate the dissemination of new variants of $bla_{\text{OXA-48-like}}$ genes. In this study we aimed to evaluate the diversity of OXA-48-like class D carbapenemase encoding genes in aquatic systems. In order to attain a more broad assessment of gene variety in these environments, we applied a culture-independent approach.

3.5.2 MATERIALS AND METHODS

3.5.2.1 Sample collection and environmental DNA extraction

Samples were collected from 3 rivers in the Vouga River basin, located in central Portugal. These rivers are highly polluted due to disposals of domestic, industrial and

agricultural origins (for more details and rivers location see Tacão *et al.* 2012). The estuarine water was collected from Ria de Aveiro, a mesothrophic estuary located in the same basin and highly polluted due to the presence of harbor facilities, aquaculture ponds, industrial plants, diffuse domestic sewage inputs and run-off from agricultural fields (Azevedo et *al.* 2012; Henriques et *al.* 2006). Water was collected into sterile bottles from 40-50cm below the water surface, and kept on ice for transportation.

Environmental DNA was purified by filtering 200-500 mL of water through 0.2-µm-pore-size filters (Poretics Products). Cells were washed from the filter with TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) followed by centrifugation (13,000 rpm, 10 min.). The pellet was ressuspended in 200 µl TE buffer enclosing 10 mg/ml of lysozyme, followed by 1h incubation at 37 ºC, and then frozen in liquid nitrogen and thawed three times. DNA extraction continued by using the Genomic DNA Extraction Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Purified DNA was stored at -20ºC.

3.5.2.2 Amplification of blaOXA-48-like gene fragments by PCR

The *bla*$_{\text{OXA-48-like}}$ gene fragments were amplified from a pool of environmental DNA from rivers and from DNA extracted from estuarine water with the two *bla*$_{\text{OXA-48-like}}$-specific primer sets described so far, designed by: (i) Poirel *et al.* (2011) (fwd: 5'-GCGTGGTTAAGGATGAACAC and rev: 5'-CATCAAGTTCAACCCAACCG) and (ii) Zong (2012) (fwd: 5' AGCAAGGATTTACCAATAAT and rev: 5' GGCATATCCATATTCATC). The PCR reaction mixtures (25 µL total volume) consisted of 6.25 µL NZYTaq 2x Green Master Mix (2.5 mM MgCl2; 200 µM dNTPs; 0.2 U/µL DNA polymerase) (NZYtech, Portugal), 16.25 µL of ultrapure water, 0.75 µL of each primer (reverse and forward), and 50-100 ng of purified DNA. PCR reactions were performed in a MyCycler Thermal cycler (Bio-Rad, USA) with conditions as described by Poirel *et al.* (2011) and Zong (2012). Positive and negative controls were included in each PCR reaction. Water was used as negative control and a *Shewanella xiamenensis* strain carrying a *bla*$_{\text{OXA-48}}$ gene was used as positive control (Tacão *et al.* 2013). PCR products

were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

3.5.2.3 Genomic library construction and analysis

Clone libraries of *bla*$_{\text{OXA-48-like}}$ gene fragments were constructed using the TA Cloning Kit, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and *Escherichia coli* NZYStar competent cells (NZYTech, Portugal). Clones were screened by PCR for the presence of fragments with the expected size, using primers targeting the vector (T7 forward: 5'- TAATACGACTCACTATAGGG and M13 reverse: 5'- CAGGAAACAGCTATGAC). Amplicons were purified and sequenced. Similarity searches in the GenBank database were performed using the BLAST tool with the deduced amino acid sequences. A phylogenetic tree was obtained using MEGA, version 6.0 (Tamura *et al.*2013). The Shannon index of diversity (H) was calculated for each library by using the formula $H = -\Sigma(ni/N)\log(ni/N)$, where *ni* is the abundance of each *bla*$_{\text{OXA-48-like}}$ type and N is the sum of the analyzed clones in each library.

3.5.2.4 Nucleotide sequences

All the nucleotide sequences obtained in this work have been deposited in the GenBank database under the accession numbers KJ620426 - KJ620480.

3.5.3 RESULTS AND DISCUSSION

In this study we evaluated the diversity of *bla*$_{\text{OXA-48-like}}$ genes in river and estuarine water by culture-independent methodologies.

From river water DNA it was possible to amplify *bla*$_{\text{OXA-48-like}}$ genes using the two primers sets, and both amplicons were used for constructing two libraries. From estuarine water DNA a amplification was obtained using the primer set described by Poirel and

coworkers (2011), and only this amplicon was used. Overall, three clone libraries of *bla*$_{\text{OXA-48-like}}$ genes were constructed.

A total of 145 inserts with the expected size were sequenced: 75 from the river water library (35 amplified with primers described by Zong 2012 and 40 with primers described by Poirel *et al.* 2011) and 70 from the estuarine water library. Gene libraries from river water comprised 35 *deduced amino acid sequence* variants (H = 1.23), from which 31 corresponded to new amino acid sequences and 4 were 100% identical to previously described sequences (i.e. OXA-48, OXA-181, OXA-199 and OXA-204). Both primer sets detected blaOXA-48 sequences which were the most abundant in both libraries, in a total of 70 clones. Ten variants were only detected by the primer set of Zong (2012) and 19 variants were exclusively detected by the primer set designed by Poirel *et al.* (2011).

The estuarine water library encompassed 22 amino acid sequence variants (H = 0.71), 19 of which were new and 3 have been already reported (100% identical to OXA-48, OXA-162 and OXA-199).

In total, deduced amino acid sequences obtained from 70 clones were 100% identical to OXA-48 (25 sequences from riverine water and 45 from estuarine water). Thirteen variants were detected in 2 or more clones and the remaining sequences (45) were detected only once in the gene libraries. Overall, 50 new variants were detected, with 1 to 3 amino acid differences from OXA-48. Besides OXA-48 and OXA-199, only two other variants were common to both the river and estuary libraries (OXA-new14 and OXA-new17).

The amino acid substitutions in the most common variants when compared to the OXA-48 sequence are shown in Figure 1. Table S1 in supplemental material indicates the amino acid substitutions in all new OXA-48-like variants found.

Noteworthy the OXA-48 variants found more frequently were those that are already triggering serious health concerns in several hospital settings, which is the case of OXA-48 that was by far the most frequently detected in both libraries. These results suggest that there might be a correspondence between what has been observed so far in hospital sets and the environmental *bla*$_{\text{OXA-48}}$ gene pool. If this is the case, new variants here frequently detected, might also emerge in clinical settings.

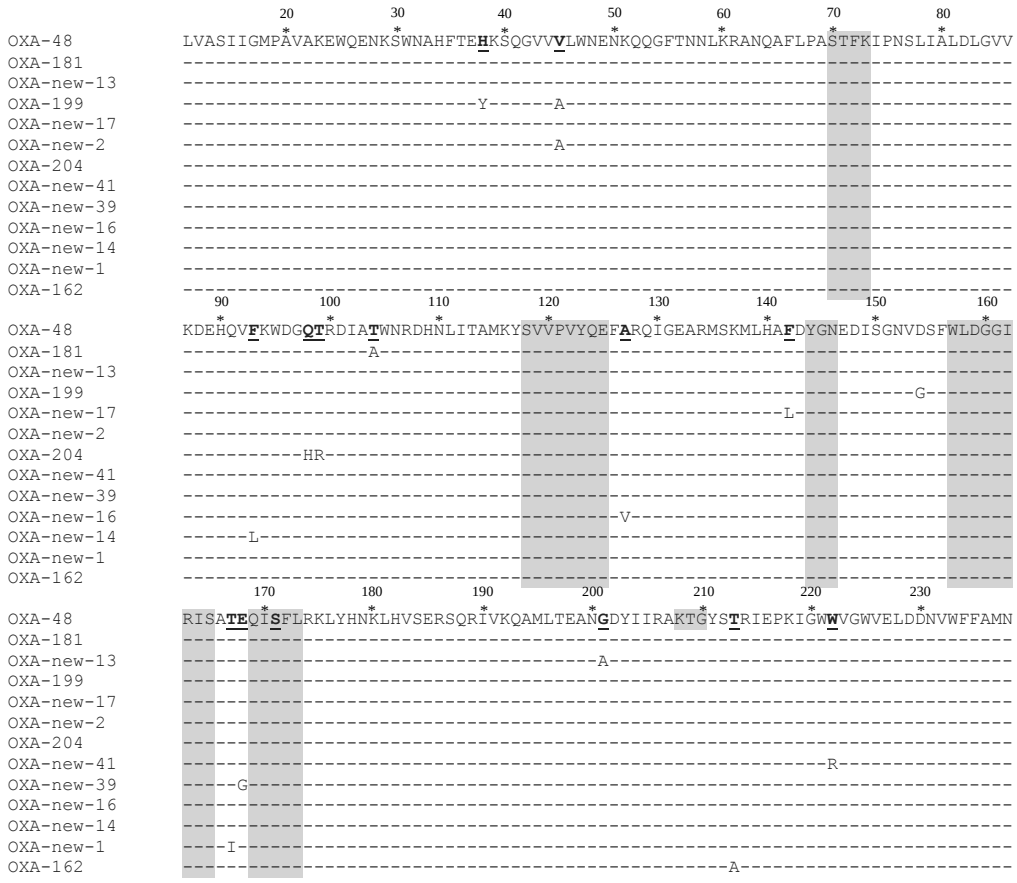

FIG. 1: Deduced amino acid sequence alignment of OXA-48 and the other more abundant variants found (identified in 2 or more clones). Dashes indicate identical residues among all the amino acid sequences. Amino acid motifs that are conserved among class D beta-lactamases are indicated by boxes in grey. Numbering is according to the class D beta-lactamase system (DBL) (Couture *et al.* 1992).

Figure 2 shows a maximum-likelihood dendrogram of representatives of OXA beta-lactamase families and the deduced amino acid sequences detected in this study in two or more clones. Sequences obtained in this study clearly affiliated with previously described OXA-48-like class D carbapenemases.

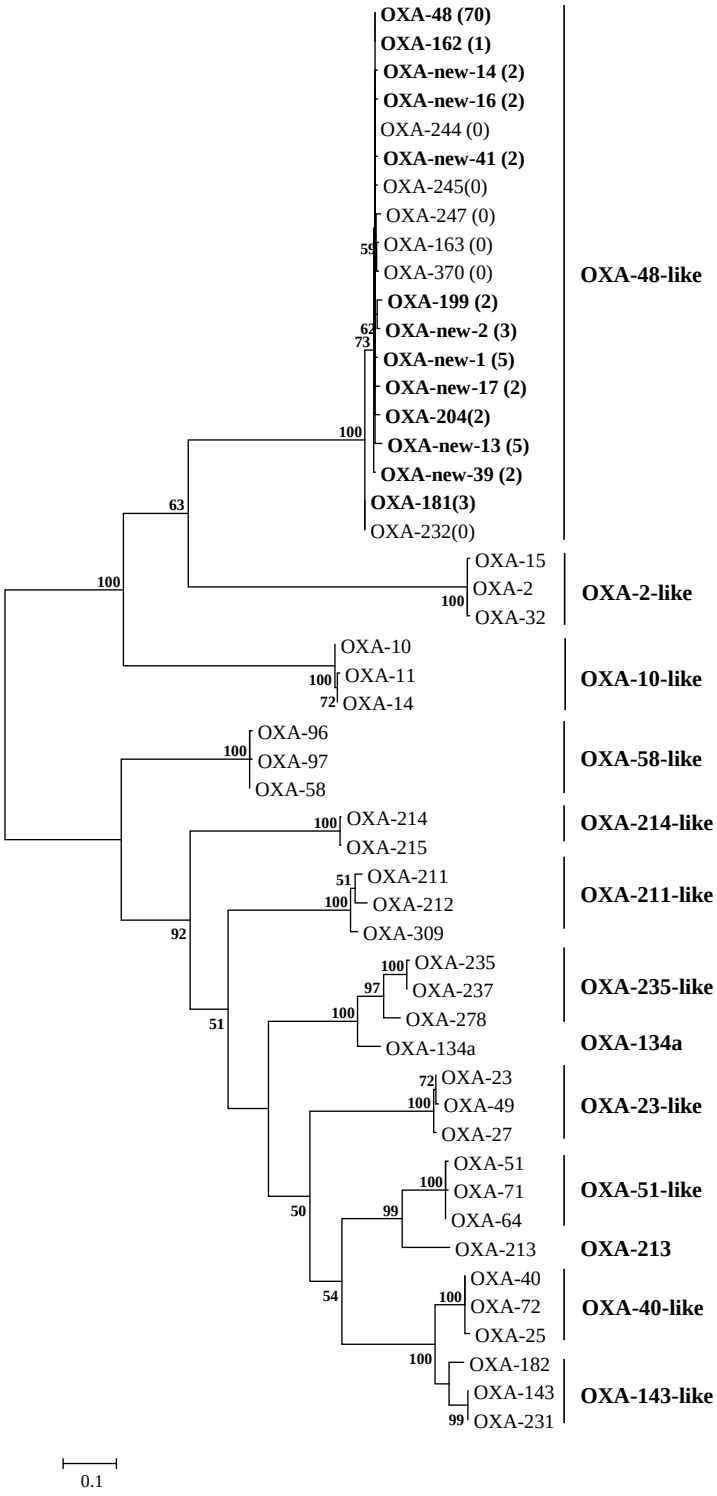

FIG. 2: Maximum-likelihood tree based on deduced amino acid sequences of representatives of OXA beta-lactamases families (OXA-2-, OXA-10-, OXA-23-, OXA-40-, OXA-48-, OXA-51-, OXA-58-, OXA-134a-, OXA-143-, OXA-211-, OXA-213-, OXA-214-, and OXA-235-like) and OXA-48-like sequences identified in 2 or more clones retrieved from gene libraries constructed in this study. Numbers in parentheses indicate the number of times that the sequence was found in the libraries. The branch number refers to the percent confidences as estimated by a bootstrap analysis with 1,000 replications.

The aquatic environments here analyzed are impacted by different pollution sources, of domestic, industrial and agricultural origins (Azevedo *et al.* 2012, Henriques et *al.* 2006; Tacão *et al.* 2012). Previously reported studies confirmed polluted aquatic systems as reservoirs and places for the evolution of antibiotic resistance (Tacão *et al.* 2012). Besides, different strains of *S. xiamenensis* have been previously isolated from the sampling sites included in this study carrying $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-204}}$ genes (Tacão *et al.* 2013).

Few studies assessed the presence of OXA-48-like-producers in environmental settings, and so it is not possible to clarify if the gene diversity here described is particular to these aquatic systems or if these genes are more diverse than expected and commonly present in environmental compartments worldwide. As the putative origin of this gene is attributed to *Shewanella* spp., commonly found in aquatic environments, this later hypothesis seems plausible.

Most probably, diverse mobilization events have mediated the transfer of $bla_{\text{OXA-48-like}}$ genes from *Shewanella* spp. to Enterobacteriaceae or other still unidentified hosts which reinforces the importance of these environmental compartments in the evolution and spread of antibiotic resistance. In fact, there are already reports of Enterobacteriaceae members isolated from river (Potron *et al.* 2011) and wastewater (Galler *et al.* 2013) carrying $bla_{\text{OXA-48}}$ genes. Mobilization may have been mediated by diverse mobile genetic platforms, previously linked to $bla_{\text{OXA-48-like}}$ genes (Poirel *et al.* 2012). These include IncA/C, Inc F-like and Inc L/M and plasmids, but also the ColE-type plasmids which are non-conjugative but mobilizable (Poirel *et al.* 2012, Sidjabat *et al.* 2013, Sampaio *et al.* 2014).

The hypothesis that the environmental gene pool detected in this study may be a result of clinical-related contamination is far less probable since, until now, no $bla_{\text{OXA-48-like}}$ producer was identified in Portuguese clinical settings. This might be related to the national carbapenems prescription policies and awareness campaigns (Henriques *et al.* 2012). However, on a worst case scenario, in the particular case of $bla_{\text{OXA-48}}$ genes, what might be happening is a silent spread, i.e., undetectable due to the low level resistance to carbapenems.

By applying environmental DNA-based methodologies both culturable and unculturable fractions are covered. However, it is important to acknowledge that by using PCR-based methodologies, the diversity found is biased by the primer sets used, which were designed based on previous described sequences. In the case of this study it was confirmed that different primer sets assessed different sequence variants, thus highlighting the need to improve the molecular-based strategies of $bla_{\text{OXA-48}}$ detection. Besides, culture independent approaches may detect DNA sequences that do not encode active beta-lactamases.

Nevertheless, the molecular approach here applied added relevant information to the current knowledge on the diversity of OXA-48-like carbapenemases.

3.5.3 CONCLUSIONS

The diversity of OXA-48-like sequences identified by culture-independent methods indicates that the environment and in particular aquatic systems constitute important reservoirs of these genes. Also, from this study resulted a list of diverse variants of OXA-48 genes that should be taken into account when designing molecular strategies for detecting this gene. As the diversity of $bla_{\text{OXA-48-like}}$ resulting from using different primer sets differed, it is advantageous to use more than one set of primers to accurately characterize any given sample.

The observation of such a diverse $bla_{\text{OXA-48-like}}$ gene pool in these aquatic systems indicates the need of further research in at least 3 lines: identification of the host species, assessment of expression and activity of the gene products, and evaluation of the capability of dissemination among strains of the variants here reported. Even so, these observations may represent a forewarning of $bla_{\text{OXA-48-like}}$ genes dissemination.

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SUPPLEMENTAL MATERIAL

TABLE S1: Amino acid substitutions and positions in all OXA-48 variants identified. Numbering is according to class D beta-lactamase system

OXA	21	30	33	35	45	72	79	82	92	93	98	99	104	113	120	125	127	131	132	136	140	141	142	144	149	152	154	158	159	167	168	169	170	172	178	183	187	194	195	201	205	209	211	212	222	226	227	229	234	248
-48	V	S	A	F	V	F	I	D	V	F	Q	T	T	T	V	E	A	G	E	S	H	A	F	Y	I	N	D	L	D	T	E	Q	I	F	H	V	S	A	M	G	I	T	Y	S	W	V	E	D	F	G
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OXA	21	30	33	35	45	72	79	82	92	93	98	99	104	113	120	125	127	131	132	136	140	141	142	144	149	152	154	158	159	167	168	169	170	172	178	183	187	194	195	201	205	209	211	212	222	226	227	229	234	248
-48	V	S	A	F	V	F	I	D	V	F	Q	T	T	T	V	E	A	G	E	S	H	A	F	Y	I	N	D	L	D	T	E	Q	I	F	H	V	S	A	M	G	I	T	Y	S	W	V	E	D	F	G
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4

FINAL CONSIDERATIONS

4.1 MAIN CONCLUSIONS

From economic and social viewpoints, multiple factors have been highlighted as drivers for the global antibiotic resistance expansion (Shallcross and Davies 2014). Far more frequently acknowledge as a central feature impelling increasing levels of global antibiotic resistance is the abusive use of antibiotics in clinical settings. In this way, these are well recognized hotspots for the acquisition and dissemination of genetic determinants of antibiotic resistance. Nevertheless, the range of scenarios where this selective pressure is exerted is beyond clinical institutions (Wellington *et al.* 2013).

For many years the study of antibiotic resistance among human pathogens has absorbed the large majority of investigations. This research focused on the consequences to human health that upsurge from the increasing prevalence of antibiotic resistant microorganisms, with the consequent inefficacy of relevant drugs used for treating serious infections. In recent years there have been an increasing number of studies on resistance associated to bacteria present in natural habitats. Several aspects justify this recent interest:

i) many environmental microorganisms are antibiotic producers, thus carrying antibiotic resistance mechanisms and contributing for their development on adjoining bacteria (Baquero *et al.* 2008, Baquero *et al.* 2009, D'Costa *et al.* 2011).

ii) antibiotic resistance can be found even in remote locations or untouched environments, where no direct selective pressure is identifiable (Aminov 2010, D'Costa *et al.* 2011) suggesting that there are no antibiotic-free environments on Earth (Allen *et al.* 2010)

iii) it has been shown that currently known genetic determinants of resistance encoded other functions in the cell (including antibiotic biosynthesis), which later turned useful for dealing with these drugs (Baquero *et al.* 2009, Martinez 2009a).

iv) the putative origin of several clinically-relevant genetic determinants of resistance has been linked to environmental bacteria (Poirel *et al.* 2002, Poirel *et al.* 2004)

v) environmental compartments are continuously influenced by human activities, including discharges from diverse sources. Those include antibiotics, antibiotic-resistant bacteria, antibiotic resistance genes and other contaminants that may co-select for antibiotic resistance (Wellington *et al.* 2013).

Particularly aquatic settings constitute large reactors where the spread, dissemination and maintenance of antibiotic resistance may be facilitated (Allen *et al.* 2010, Lupo *et al.* 2012, Taylor *et al.* 2011). Aquatic environments such as rivers, streams or lakes:

- are impacted by different elements merged in agricultural, domestic and industrial discharges;
- accumulate antibiotics and other compounds, such as detergents or heavy metals, that may persist for long periods;
- collect pathogenic and non-pathogenic bacteria, antibiotic resistant bacteria and antibiotic resistance genes, incoming from different origins;
- allow the mixing of incoming bacterial populations with the resident antibiotic producers and/or bacteria intrinsically resistant to antibiotics.

For these reasons, studying the resistome of this particular environmental compartment is essential to further elucidate the role of human activities in the dissemination and persistence of antibiotic resistance, particularly in what concerns antibiotics used for treating serious infections caused by Gram-negative bacteria. Hence, in this study we have focused on resistance towards 3rd generation cephalosporins and carbapenems that are critically important for human health and are used in many cases as final treatment options for dealing with infections caused by multiresistant strains.

Over the years the prevalence of bacteria resistant to these last-line drugs has been continuously increasing, compromising their efficiency. Although there have been some reports focused on bacteria resistant to 3rd generation cephalosporins detected in some aquatic compartments, reports dedicated to carbapenem resistance in this particular environmental settings are scarce.

In this way we have established the hypothesis that aquatic environments, particularly rivers, are reservoirs and diffusers of antibiotic resistance and that human activities promote these events. To test this hypothesis, we set as main goal to characterize and compare the environmental antibiotic resistome in polluted and unpolluted river water, particularly in what concerns resistance towards last-resort antibiotics. The study focused the hydrographic region of Vouga River basin, which encompasses aquatic settings exposed to different anthropogenic impacts.

Taking into account that less than 1% of environmental bacteria are culturable (Allen *et al.* 2010), we have used culture-dependent coupled with culture-independent methodologies to extend our knowledge on the antibiotic resistance profile of the microbial community present in these particular environments.

Globally, several key observations contributed for sustaining the above mentioned study hypothesis. Particularly it was possible to state the following main conclusions:

1- <u>Rivers are reservoirs and disseminators of last-resort antibiotic resistance determinants</u>

To assess the role of rivers as reservoirs of antibiotic resistance to last-resort antibiotics, two bacterial collections were established comprising: 1) bacterial strains selected in culture media containing cefotaxime and 2) bacterial strains selected in culture media supplemented with imipenem. These culture collections were evaluated in what concerns phylogenetic diversity and antibiotic resistance phenotypes and genotypes.

Both cefotaxime- and imipenem-resistant collections included a wide diversity of Gram-negative bacteria. We found that the phylogenetic groups that apparently play a relevant role in the dissemination of antibiotic resistance in these environmental settings include Enterobacteriaceae members (most probably related to faecal pollution), *Pseudomonas* spp. that often presented high levels of resistance to last-resort antibiotics and *Aeromonas* spp. (**chapter 3.1 and chapter 3.3**).

Results showed that cefotaxime resistance was frequently associated to the production of extended-spectrum beta-lactamases, while carbapenems resistance was mostly related to intrinsic mechanisms such as the production of chromosomally-encoded carbapenemases (**chapter 3.1, chapter 3.2, and chapter 3.3**).

Moreover, clinically-relevant resistance mechanisms were identified among isolates from both cefotaxime- and imipenem- resistant bacteria collections, predominantly in bacteria isolated from polluted water. These included *bla*$_{\text{CTX-M-like}}$, *bla*$_{\text{OXA-48-like}}$ and *bla*$_{\text{VIM-2}}$ genes (**chapter 3.1, chapter 3.3, chapter 3.4**), which have been already linked to bacterial strains causing serious infectious diseases outbreaks worldwide (Cantón *et al.* 2012, Patel and Bonomo 2013, Poirel *et al.* 2012).

Several evidences were gathered indicating that co-resistance mechanisms are frequent in riverine bacteria. Co-resistance mechanisms identified in this study included: (i) one resistance gene encoding resistance to different classes of antibiotics (e.g. *aacA4-cr* genes that encode resistance to aminoglycosides and fluoroquinolones); (ii) several resistance genes in the same genetic platform (e.g. integrons gene cassettes arrays); and (iii) one resistance gene encoding resistance towards all antibiotics included in one class (e.g. resistance to all fluoroquinolones due to mutations in the topoisomerase gene *parC*) (**chapter 3.1, chapter 3.2**). Under antibiotic selective pressure these co-resistance mechanisms give an advantage to the microorganism and imply that limited therapeutic options would be available for the treatment of infections caused by these strains (**chapter 3.2**). Although it would be expected that carrying several resistance genes would have an increasing fitness cost to the bacteria, it has been discussed that owing to compensatory events the presence of several resistance genes might even increase bacteria fitness (Cantón and Ruiz-Garbajosa 2011).

As described in clinical settings, multiresistance was often observed in bacteria isolated in this study (**chapter 3.1**, **chapter 3.3**). Frequently multiresistance was associated to the presence of mobile genetic elements carrying genes conferring resistance to several antibiotic classes. For example, conjugation experiments showed that the multiresistance phenotype registered for *bla*$_{\text{CTX-M}}$-producers was due to the presence of narrow host range (NHR) plasmids, such as IncF, IncK and IncI1, carrying several genetic determinants of resistance (**chapter 3.2**). Furthermore results showed higher prevalence of class 1 integrons in ESBL-producers and the presence of identical

arrays in different strains. This also suggest that integrons are exchanged and disseminated easier among $ESBL^+$ strains, playing a relevant role on the dissemination of antibiotic resistance in rivers (**chapter 3.2**). Furthermore, we have found gene cassette arrays that are frequently reported worldwide in both clinical and environmental samples (e.g. *dfrA17-aadA5*) but also new arrays (e.g. *qnrVC4 – aacA4'-17*) or genetic determinants identified in new hosts (e.g. *qnrVC4* in *Pseudomonas* sp. and *Escherichia coli*.) (**chapter 3.2**).

To evaluate risks to human health, the majority of published work regarding environmental microorganisms aim to characterize the most problematic pathogenic bacteria found in clinical settings. However, these represent a minority when compared to the large number and diversity of microorganisms and resistance genes present in the environment. Thus, the diversity of resistance mechanism residing within the environmental resistome is far from being completely disclosed. In fact, we observed by culture-independent methods that the environmental diversity of $bla_{\text{CTX-M-like}}$ and $bla_{\text{OXA-48-like}}$ gene sequences is greater than what has been reported so far in clinical settings (**chapter 3.1, chapter 3.5**).

2- <u>Anthropogenic activities modulate the riverine resistome and potentiate the dissemination of bacterial resistance to last-resort antibiotics</u>

In this study it was possible to include river waters classified as polluted and unpolluted, considering the physical, chemical and microbiological parameters established by the Portuguese law for water quality determination . This classification was crucial for the analysis of our results, as also to draw conclusions.

The prevalence of cefotaxime- and imipenem-resistant bacteria was higher in polluted water (**chapter 3.1, chapter 3.3**).

In both cefotaxime- and imipenem-resistant bacterial collections, the number of multiresistant strains was higher among isolates from polluted environments (**chapter 3.1, chapter 3.3**)

We have also observed that *bla*$_{\text{CTX-M-like}}$ gene sequences found in unpolluted water were similar to ancestral chromosomal genes while in polluted water, besides the higher diversity detected, also *bla*$_{\text{CTX-M}}$ sequences were identical to those frequently reported in clinical settings (**chapter 3.1).** These results further reinforce the relevance of water pollution in modulating the environmental resistome.

Concerning resistance to carbapenems, *bla*$_{\text{CphA}}$ and *bla*$_{\text{L1}}$ were detected in *Aeromonas* spp. and *Stenotrophomonas maltophilia*, respectively, in both polluted and unpolluted waters. However, *bla*$_{\text{VIM-2}}$ genes which constitute an example of acquired resistance to carbapenems, were detected in *Pseudomonas* sp. strains isolated only from polluted river water.

Results suggest that data on the occurrence and diversity of specific genes may be useful to assess ecosystems health and antibiotic resistance evolution. In particular, *bla*$_{\text{CTX-M}}$ genes showed good potential as pollution indicators, as also *bla*$_{\text{VIM-2}}$ genes. Source tracking methods must be conducted to link the presence of *bla*$_{\text{CTX-M}}$ or *bla*$_{\text{VIM-2}}$ genes to specific sources of contamination. Also, similar studies on other geographical sites and different environmental compartments should be performed to validate this application.

3- <u>Bacterial strains and genes previously identified as the origin of genetic determinants of resistance are present in riverine water</u>

An environmental putative origin has been indicated for some clinically-relevant resistance mechanisms, including *bla*$_{\text{CTX-M}}$ genes in environmental *Kluyvera* spp. (Poirel *et al.* 2002) and *bla*$_{\text{OXA-48}}$ genes in *Shewanella* spp. (Poirel *et al.* 2004). In this study we have also detected *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{OXA-204}}$ genes in *Shewanella xiamenensis* strains, recognized as the putative origin of *bla*$_{\text{OXA-48-like}}$ genes (**chapter 3.4**). We have found for the first time *S. xiamenensis* strains carrying a *bla*$_{\text{OXA-204}}$ gene suggesting that the emergence of different *bla*$_{\text{OXA-48-like}}$ genes probably had origin in different *S. xiamenensis* strains. Moreover, the genetic context was identical to those previously described in other environmental *Shewanella* spp. (Poirel *et al.* 2012).

Furthermore, as stated above, by culture-independent methodologies we also detected in unpolluted water putative $bla_{\text{CTX-M-like}}$ ancestral sequences. These findings also support the idea that the environmental origin of clinically-relevant resistance mechanisms is independent of human actions.

4.2 FINAL CONSIDERATIONS

When studying antibiotic resistance it is of major relevance to widen the range of target microorganisms, to include pathogenic but also non-pathogenic naturally occurring bacteria. In this context, in contrast with research focused on clinical microorganisms, studying environmental bacteria is far more challenging. There are no standard methods for isolating microorganisms or growing conditions, as culture media, incubation conditions or antibiotic concentration. Also there are no guidelines for classifying environmental bacteria as resistant or susceptible using phenotypic-based methodologies. Usually, antibiotic susceptibility tests are performed and interpreted according to recommendations given by the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST), which have been elaborated based solely on clinical microorganisms characteristics. In this case, it would be relevant to establish breakpoints for environmental microorganisms and have an accurate analysis of the resistance patterns observed in a specific environmental compartment. Variations in methodologies presented in studies reported so far invalidate comparisons between different environmental compartments or locations.

It is quite important also to expand the variety of environmental compartments to consider. So far, reports have focused mainly on characterizing microbial populations present in wastewaters/sludge and/or discharge points, and only a few were focused on rivers/lakes water or sediments. Taking into account that: (i) rivers are major collectors of wastewaters, sludge and agricultural run-offs that enclose antibiotics and other compounds, pathogenic and non-pathogenic, antibiotic resistant bacteria and genes, and (ii) river water is used for different purposes from leisure to occupational activities.

Thus surveillance measures are imperative for maintaining the usefulness and sustainability of these water habitats. Comparing results obtained at different geographic locations, establish surveillance programs and coordinate information collected in environmental compartments worldwide, from locations with restrict to more tolerant prescription policies, would also be beneficial. Planning strategies for analyzing antimicrobial resistance should consider also all the genetic units that might be involved in the maintenance and spread of antimicrobial resistance (genes, genomic context, genetic platforms, and clones).

Although many factors have been contributing to the growing rates of antibiotic resistance over the years, still the abusive and inappropriate use of antibiotics is repeatedly acknowledged as a central cause for this trend. Hence mitigation strategies must be implemented in clinical institutions but also in agriculture settings and the environment. The application of antibiotic stewardship programs is important to achieve the best clinical outcomes but still decrease the antibiotics selective pressure, in both medical and environmental settings. Limiting and/or reducing antibiotic consumption in both clinical and agricultural settings (as prophylactics or growth promoters in livestock) is crucial for maintaining the efficiency of essential drugs.

It is also important to monitor and reduce the influx of antibiotics, antibiotic resistance genes and bacteria to natural environments, through domestic and hospital wastewaters but also from agricultural run-offs.

Nowadays, the commercial production of antibiotics overcomes their natural synthesis, estimated in millions of metric tons per year (Segura *et al.* 2009). In this way, globally humans are the main contributors for the presence of antibiotics in the environment (Gillings 2013). Also the disposal of other compounds must be supervised, including heavy metals and biocides, which have been proven to contribute for selecting antibiotic resistant bacteria (Baker-Austin *et al.* 2006, Baquero *et al.* 2008). Finally, the load of antibiotic resistant bacteria and antibiotic resistance genes in natural settings must also be reduced, as well as the mix of microorganisms from different origins should be prevented.

Overall data gathered in this document indicate that water environments, particularly river water, have an important role in the spread and evolution of antibiotic resistance. Aquatic systems act as reservoirs of resistance genes that facilitate the

dissemination and mobilization of genetic platforms enclosing several resistance determinants. Moreover, results suggest that the dissemination of resistance to broad-spectrum antibiotics such as cefotaxime and imipenem may be at an earlier stage in unpolluted environments, providing the opportunity to monitor these aquatic systems and to identify the key human-derived negative impacts that reduce water quality continuously and consequently promote resistance dissemination.

Clinically-relevant genetic determinants of resistance that have already been linked to serious outbreaks worldwide were identified mostly in polluted water and in association with mobilizable genetic platforms. These observations warn for the relevance of monitoring anthropogenic activities in these water habitats. As river water is continuously used for diverse human activities, it is essential to maintain water quality and the ecosystem equilibrium.

Given that the origin of antibiotic resistance is the environmental microbiota, it seems relevant to continue exploring natural habitats in order to fully comprehend the series of events that lead to spread and dissemination of resistance to human pathogens, or even identify new genetic determinants of antibiotic resistance and anticipate future problems.

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