CATÓLICA

UNIVERSIDADE CATÓLICA PORTUGUESA | PORTO

Escola Superior de Biotecnologia

TRACKING ANTIBIOTIC RESISTANCE FROM HOSPITAL EFFLUENTS TO THE SURROUNDING ENVIRONMENT

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology, with specialization in Microbiology

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Ana Rita Boura Varela

Supervisor: ***Célia Maria Manaia Rodrigues***
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January 2016

«Great people do things before they're ready.»

Amy Poehler

Resumo

Nas últimas décadas, o papel do ambiente na disseminação de resistência a antibióticos de relevância clínica tem recebido especial atenção. Neste âmbito, as águas residuais hospitalares e municipais, onde resíduos de antibióticos e bactérias resistentes são descarregados, estão entre os mais importantes reservatórios ambientais. Porém, o seu papel na disseminação da resistência ainda está pouco compreendido. O principal objectivo deste estudo foi o de avaliar a relevância dos efluentes hospitalares na disseminação de bactérias resistentes a antibióticos e de genes de resistência no ambiente. O estudo focou-se num efluente hospitalar e nas águas residuais da estação de tratamento receptora (ETAR).

Através da análise multivariada baseada nas variações da composição das comunidades bacterianas e das populações resistentes a antibióticos cultiváveis em função das variações da concentração de resíduos de antibióticos e metais, procurou-se compreender como se interligam estas variáveis. As correlações positivas significativas observadas sugeriram que a presença dos contaminantes químicos analisados poderia estar associada a rearranjos nas comunidades bacterianas ou à selecção de populações resistentes em águas residuais, principalmente em efluentes hospitalares. O potencial das águas residuais hospitalares e municipais como reservatórios de resistência a antibióticos foi de seguida investigado, usando como modelo membros da espécie *Escherichia coli* resistentes a quinolonas.

Foi seleccionado um grupo de estirpes de *E.coli* resistentes a quinolonas, representativo de amostragens de águas residuais municipais e de ribeiras urbanas abrangendo um período de nove anos. A diversidade genética destes isolados foi avaliada com base em *multilocus sequence typing* (MLST), e esta informação foi comparada com dados dos isolados sobre a presença de determinantes genéticos associados com resistência a quinolonas e com outras características associadas a resistência. Assim, foi possível inferir sobre a ocorrência de disseminação vertical e/ou horizontal de resistência. A análise baseada em MLST demonstrou que isolados de diferentes tipos de água e datas de isolamento se agrupavam, sugerindo não só o carácter ubiquista de algumas linhagens, mas também a sua persistência em ambientes aquáticos. A indicação de disseminação vertical de resistência sugerida por estes dados foi confirmada pelo facto de que a resistência a quinolonas era essencialmente de base cromossómica. Apesar disso, a presença dos mesmos determinantes adquiridos de resistência a quinolonas e beta-lactâmicos e de

replicões plasmídicos em *E.coli* de diferentes linhagens e origens sugeriu que estas bactérias têm um papel importante na transferência horizontal da resistência em ambientes aquáticos. Esta ideia foi reforçada pela observação de que alguns destes determinantes se encontravam em elementos genéticos conjugativos. Nesta fase, a questão era se as mesmas linhagens e genes encontrados em *E.coli* de ambientes aquáticos ocorriam em outros habitats. A mesma abordagem aplicada a um grupo alargado de isolados de origens diferentes mostrou que as mesmas linhagens podiam ser encontradas em pacientes hospitalares e em gaivotas, mas não em aves de rapina de uma reserva natural, sugerindo que algumas linhagens de *E.coli* podem ser disseminadas entre ambientes com influência humana. Sendo *E.coli* uma espécie comensal em animais e humanos, o estudo de um grupo bacteriano ambiental não associado com humanos era também de interesse. A escolha recaiu sobre o género *Aeromonas* e, numa perspectiva de continuidade com o trabalho prévio, foram seleccionadas estirpes resistentes a quinolonas. Surpreendentemente, apesar da fraca associação com humanos, observou-se que entre as *Aeromonas* spp. de efluente hospitalar se encontrava uma prevalência de resistência a antibióticos superior às da ETAR. O papel desta espécie como vector de disseminação de resistência foi evidenciado de várias formas. Primeiro, foi possível identificar em *Aeromonas* spp. do efluente hospitalar um gene de beta-lactamase previamente não descrito em *Aeromonas* spp. nem fora do contexto clínico. Segundo, viu-se que possuíam dois genes de resistência adquirida a quinolonas, um claramente ubíquo e outro de origem não clínica, o que pode ser interessante em estudos de fontes de dispersão de resistência. Terceiro, alguns dos genes de resistência a antibióticos podiam ser transferidos para *E.coli* por conjugação. Confirmou-se que *Aeromonas* spp. podem ser veículos relevantes de resistência, e que o hospital pode ser uma fonte importante de membros deste género.

A transferência horizontal de genes por conjugação e o efeito de concentrações sub-inibitórias de antibióticos são temas chave para a compreensão da disseminação da resistência a antibióticos. Este tema foi investigado com base numa estirpe multiresistente de *E.coli* de efluente hospitalar. Observou-se que enquanto uma concentração sub-inibitória de ceftazidime aumentava significativamente a taxa de conjugação, tetraciclina produzia o efeito oposto. O plasmidoma conjugativo, que em média foi transferido em mais de 90% dos ensaios, incluía possíveis determinantes de persistência, de resistência a antibióticos e a metais, sugerindo a importância de fenómenos de co-selecção.

Confirmou-se portanto que o efluente hospitalar é uma fonte relevante de bactérias resistentes a antibióticos e de determinantes de resistência para o ambiente.

ABSTRACT

Over the last decades the environment has received particular attention regarding its role on the dissemination of antibiotic resistance of clinical relevance. Hospital and municipal wastewater, where antibiotic residues and resistant bacteria are discharged, are among the most obvious reservoirs. However, their role on the resistance dissemination is still poorly understood. The primary goal of this study was to assess the role of hospital effluent in the dissemination of antibiotic resistant bacteria and antibiotic resistance genes in the environment. The focuses of the study were a hospital effluent and the wastewater of the receiving urban wastewater treatment plant.

Multivariate analyses based on the variation of the composition of the bacterial communities and prevalence of antibiotic resistant culturable populations in function of variations on the concentration of antibiotic and metal residues were performed in order to search for possible correlations among these variables. The significant positive correlations observed were a possible indication that the presence of the analyzed chemical contaminants could be associated with rearrangements of the bacterial communities or selection of resistant populations in wastewater, mainly in hospital effluent. The potential of hospital and municipal wastewater as environmental reservoirs of antibiotic resistance was, thus, further investigated, using quinolone resistant *Escherichia coli* as model.

A group of quinolone resistant *E. coli* strains representative of sampling events from municipal and hospital wastewater and urban streams, spanning a period of nine years, was selected. The genetic diversity of these strains was assessed based on multilocus sequence typing (MLST) and this information was compared with data onthe isolates regarding the presence of genetic determinants associated with quinolone and with other resistance-associated traits. In this way, it was possible to infer about the occurrence of vertical and/or horizontal resistance dissemination. The MLST-based studies showed that strains from different types of water and isolation dates clustered together, suggesting not only the ubiquitous character of some lineages but also their persistence in aquatic environments. The indication of vertical resistance dissemination that these data suggested was confirmed by the fact that quinolone resistance was mostly chromosome-based. However, acquired genetic determinants of resistance to quinolones, to beta-lactams and plasmid replicons, found in the different *E. coli* lineages and origins, suggested that these bacteria play an important role on the horizontal transfer of resistance in aquatic environments. Indeed,

some of these determinants were observed to be located in conjugative mobile genetic elements. At this stage, the question was if the same lineages and genes found in *E. coli* from aquatic environments could be found in other habitats. The same approach, applied to an extended set of isolates from different origins, showed that the same lineages could be found also in hospital patients and urban gulls, although not in birds of the prey from a natural reserve, suggesting that at least some *E. coli* bacterial lineages can be disseminated among the human-impacted environments.

While *E. coli* is an human and animal commensal species, the study of an environmental bacterial group not associated with humans was also of interest and *Aeromonas* spp. were the choice, and, as a common point, quinolone resistant strains were selected. Surprisingly, in spite of the loose association with humans, *Aeromonas* spp. from hospital effluent showed higher antibiotic resistance prevalence among them than those from urban wastewater. The role of members of this species as vectors of resistance dissemination was evidenced in different ways. First, *Aeromonas* spp. from hospital effluent were found to harbor a beta-lactamase gene not previously described in *Aeromonas* spp. or outside the clinical settings. Second, they harbored acquired quinolone resistance genes that showed either ubiquitous distribution or a non-clinical origin, both interesting tools for source tracking surveys. Third, some of their antibiotic resistance genes could be transferred to an *E. coli* receptor via conjugation. *Aeromonas* spp. were confirmed as relevant carries of resistance and the hospital can be an important source for members of this genus.

Conjugation and the effect of sub-inhibitory concentrations of antibiotics are two key issues in the understanding of the dissemination of antibiotic resistance, herein investigated based on a multidrug resistant *E.coli* isolate from hospital effluent. While a sub-inhibitory concentration of ceftazidime was observed to significantly increase the conjugation rate, tetracycline had the opposite effect. The conjugative plasmidome, which on average was transferred in more than 90% of the assays, included genes putatively associated with persistence, resistance to antibiotics and to metals, suggesting the importance co-selection phenomena.

The hospital effluent was therefore confirmed as a significant source of antibiotic resistant bacteria and antibiotic resistance determinants to the environment.

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Introduction

Water is essential for life. It is indispensable for cellular activities at several levels, from protein and DNA folding to the maintenance of cell osmotic balance (Chaplin 2006). Due to its properties, water easily permeates most of the environmental compartments, being an excellent vehicle for the interchange of substances and organisms between ecosystems. It is also one of the most essential natural resources to human life. Besides its immediate utilization for drinking, cooking and hygiene, water is directly or indirectly used in the many activities that sustain the human society in its current form, such as agriculture, animal husbandry, industrial production, transportation and electricity generation. As a result of this dependency, aquatic environments are strongly influenced by human activities. Population growth has turned water availability and quality into central issues, increasing the concern over the sustainability of water resources for human consumption and industrial/agricultural processes.

Although more than 70% of our planet is covered with water, it is estimated that only a small fraction (2.5%) is adequate and available for human use (Shikiomanov 1993). Nevertheless, the utilization rate of potable water resources has increased exponentially in the last decades (Food and Agriculture Organization of the United Nations 2011). This situation has led, mainly in some world regions, to regard wastewater as a resource, with the reuse of treated wastewater constituting an adequate solution to mitigate water scarcity, mainly in agriculture (Hanjra et al. 2012; World Health Organization 2013). While, at the present, the access to potable water is a problem essentially in developing countries, water scarcity is, in general, an issue in industrialized countries, where fast population and societal development have led to the decline of fresh water resources, either due to the intense abstraction and/or due to contamination with the discharge of effluents resulting from human activity (Food and Agriculture Organization of the United Nations 2011; World Health Organization 2015). Due to population and industrial growth, the widespread discharge of wastewater means that, nowadays, indirect reuse (*i.e.* drinking or irrigation water abstraction downstream from the discharge point of wastewater treatment facilities) may occur, further increasing the need to ensure that wastewater is properly treated. In the face of these circumstances, the need to reformulate the global water management strategy has been addressed by several national and international authorities (Directive 2000/60/EC 2000; Palaniappan et al. 2010; World Health Organization 2013).

1. Wastewater - the current situation

1.1 Origins and types

Wastewater can be classified according to its origins, which may be domestic, industrial, resulting from the agriculture and animal production, run-off from rain and soil leachates, among several others (Henze & Comeau 2008). Depending on the origin, wastewater may differ in chemical composition, containing distinct types of residues and environmental contaminants. For example, while domestic wastewater is likely to transport residues from household and personal care products and from the human metabolism (black wastewater), industrial wastewater will have a composition that reflects the industry from which it is originated. Industrial wastewater which is discharged into municipal urban wastewater treatment plants frequently undergoes a preliminary treatment, as municipal collectors are only supposed to receive and treat discharges whose composition meets the definition of domestic effluent. In addition, in combined sewer systems, storm water is also directed to the treatment plant.

Socio-economic status and demographic issues, and in particular population density, are also known to influence the characteristics of domestic wastewater. Population growth and technological development, which has given rise to new classes of xenobiotic compounds unknown to natural environments (Pal et al. 2014), have contributed for an increased complexity in the composition of wastewaters. Contaminants of emerging concern (CEC), whose possible adverse effects for the environment and human health have just recently begun to be documented, are in most cases not yet covered by any regulation (Verlicchi et al. 2010; Petrie et al. 2014).

Hospital effluents, despite representing a potential source of chemical and biological hazards, are, in general, classified as domestic effluents and not contemplated under specific legislation (Carraro et al. 2016). Therefore, and although some hospitals have dedicated treatment systems, in most world regions there is no legal requirement for this procedure and untreated hospital effluents can be discharged directly into the municipal wastewater treatment facility (e.g. Code of Federal Regulations 40 2014; Directive 91/271/EEC 1991; Carraro et al. 2016). Although the load of hospital effluent is generally much smaller in volume than the total of the domestic effluent received by a municipal wastewater treatment, hospitals may constitute a privileged source of substances like antibiotics, disinfectants, diagnostic agents and anesthetics (Verlicchi et al. 2010), and of clinically relevant microorganisms like pathogenic and/or multidrug resistant bacteria

(Korzeniewska et al. 2013; Alexander et al. 2015), which will integrate wastewater bacterial communities.

The wastewater microbiota, which is composed by a wide variety of microbial groups, combines those that naturally inhabit water environments and those discharged from human activities. These include viruses, bacteria, or eukaryotic microbes such as amoebas or plasmodium (Mara et al. 2003). The diverse roles of some bacterial representatives of these communities will be further discussed in the following sections.

1.2 Bacterial populations in wastewater

Typical indigenous inhabitants of wastewater include microorganisms that are able to use the components of wastewater to obtain nutrients for growth and reproduction. This is carried out by organoheterotrophs through the decomposition of organic molecules, either through aerobic or anaerobic respiration or fermentation; and by nitrifying or anammox bacteria through the oxidation of inorganic nitrogen reduced compounds, and of other lithotrophs and phototrophs through the oxidation of iron, sulfur or hydrogen that use mineral electron donors or light as energy sources. In terms of taxonomy, the predominant bacterial groups in wastewater are the same that can be found in other types of water, and include members of the phyla *Proteobacteria* (typically of the classes *Alpha*-, *Beta*- and *Gammaproteobacteria*), *Actinobacteria*, *Bacteroidetes* and *Firmicutes* (Vaz-Moreira et al. 2014).

Other bacterial inhabitants of wastewater include those with origin in sanitary wastes and soil leachates, mostly animal and human commensals or pathogens. Bacteria of enteric origin, such as Escherichia coli or enterococci, are typical inhabitants of the gut of warm blood animals and therefore can be found in wastewater as a result of their fecal discharge. The fact that these bacteria are not expected to multiply in water has weighted on their selection as suitable indicators of fecal contamination (Mara et al. 2003). The concept of microbiological indicator, which includes also other bacteria of non-fecal origin such as Pseudomonas aeruginosa or sulfite reducing clostridia, is very useful, since the screening of all potentially hazardous human and animal pathogens would be unpractical or impossible. Although indicator bacteria are often not harmful for humans, some members of these species can behave as opportunistic pathogens, through infection or exotoxin production, being related for instance with hemorrhagic colitis or hemolytic uremic syndrome (e.g. Castro-Rosas et al. 2012; Saxena et al. 2015). The usefulness of indicator bacteria relies in the fact that fecal contamination in water can be used as a proxy for the

risks of occurrence of some important waterborne illnesses, such as cholera, salmonellosis and leptospirosis, among others (Mara et al. 2003; Saxena et al. 2015). The definition of maximum admissible levels of indicator bacteria in water (Council Directive 98/83/EC 1998; World Health Organization 2011) is therefore a major tool to assure water microbiological quality and safety.

1.3 Wastewater treatment – an overview

The complexity of wastewater treatment has increased in parallel with the scientific and technological development of the last decades. Since the beginning of the 20th century, wastewater treatment has seen the introduction of different treatment stages to gradually reduce the amount of organic matter (mainly in the form of suspended solids), phosphates, ammonia and nitrates entering the environment (Tchobanoglous et al. 2003), in order to produce a final effluent with minimal impact on natural water bodies. The selection of a specific wastewater treatment process depends on the type of wastewater, country, climate, costs, technology available and destination of the treated effluent, among other factors (Mateo-Sagasta et al. 2015).

Wastewater processing usually starts with a preliminary treatment stage of filtration where the larger residues are removed. This is followed by a primary treatment, consisting on the physical removal of suspended particles through sedimentation in proper tanks, which may be aided by the addition of chemicals that promote particle settling. Secondary treatment is aimed at the degradation of organic matter and removal of phosphorous and nitrogen. This stage of the treatment is constituted mainly by biological processes, taking advantage of metabolic functions of microorganisms, such as organic biodegradation or nitrogen conversion. This step of the treatment can be either predominantly aerobic or anaerobic, and performed in systems where the microorganisms are either fixed to a surface (e.g. trickling filter or biological aerated filter) or suspended in the water (e.g. activated sludge). In many wastewater treatment plants, this is the final treatment step before the effluent is released into the environment. In other cases, a tertiary treatment is included. The tertiary treatment aims at the further cleaning of wastewater through the removal of components that were not removed in previous phases, such as residual suspended solids, pathogens and protozoan parasites. This form of treatment may include sand filtration and disinfection with UV and/or O_3 or chlorine (Helmer et al. 1997; Mara et al. 2003; Tchobanoglous et al. 2003; Okoh et al. 2007; Henze & Comeau 2008).

In order to be released in the environment, treated wastewater must comply with the levels defined by law for parameters such as biological oxygen demand, chemical oxygen demand, phosphorous, nitrogen and other substances (Table 1). In the European Union all legislation regarding drinking water quality and wastewater treatment has been aggregated under the Water Framework Directive (Directive 2000/60/EC 2000), in order to regulate water policies among its members and assure water quality and sustainably. A similar effort has been carried out in the United States of America, where the regulations regarding water pollution and wastewater are collected under the Clean Water Act (United States Environmental Protection Agency).

2. Human health and environmental risks associated with wastewater

The major concern behind wastewater treatment is related with potential risks for human health. In regions with inadequate or inexistent wastewater management, such as low income countries, the main risk for human health associated with wastewater is the potential for contact with pathogens (World Health Organization & UNICEF 2012). In countries with higher income and technological development, the main risks associated with wastewater refer to CEC, such as pharmaceutical products or pollutants resulting from industrial activities which may be not extensively removed during wastewater treatment (World Health Organization & UNICEF 2012). Examples of pharmaceutical contaminants in wastewater include substances from various therapeutic classes (e.g. antibiotics, hormones, anti-inflammatory drugs, anti-depressants, anesthetics), personal care (e.g. sunscreen) and household products (e.g. disinfectants), while other relevant contaminants include the residues from newly developed materials that have been recently introduced in routine daily life (e.g. flame retardants) (Pal et al. 2014; Petrie et al. 2014). If not removed by the wastewater treatment, these contaminants can enter the water bodies and be mobilized to different environmental compartments, including rivers, oceans, underground aquifers and soils (Figure 1). Hypothetically, these contaminants can enter the human food chain, either directly due to abstraction of contaminated water for drinking purposes, or indirectly, due to plant uptake of contaminants from irrigation water. The effect of these contaminants in human health, alone or combined (synergistic effect), is yet to be understood (Covaci et al. 2012; Pal et al. 2014).

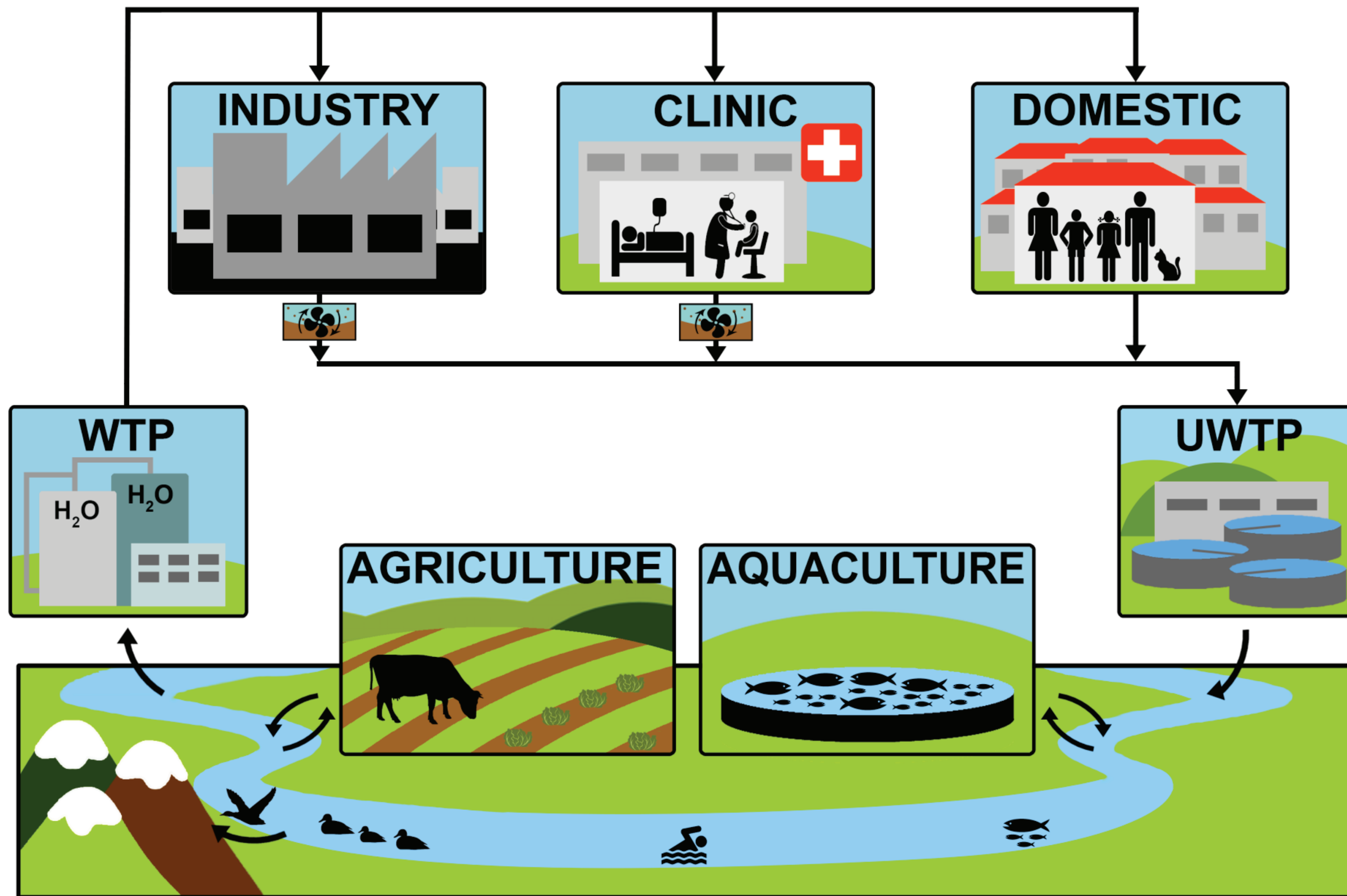

Figure 1 - Circulation of bacteria over urban and environmental water compartments. WTP , water treatment plant; UWTP, urban wastewater treatment plant

Table 1 - European legislation regarding wastewater and hazardous substances discharge into environmental water bodies

Type of wastewater	Origin	Requirements for discharges	Legislation
Urban wastewater	Domestic waste water from residential settlements and services which originates predominantly from the human metabolism and from household activities Run-off rain water	to water bodies in general BOD_5<25 mg/L O_2, without nitrification, 70-90% reduction relative to the influent COD<125 mg/L O_2, 75% reduction relative to the influent TTS<35 mg/L[a], 70-90% reduction relative to the influent to sensitive areas[d] which are subject to eutrophication Total phosphorus<2 mg/L[b] or < 1 mg/L[c] with a 80% reduction relative to the influent Total nitrogen<15 mg/L[b] or <10 mg/L[c] (or a daily average< 20 mg/L) with a 70-80% reduction relative to the influent	Directive 91/271/EEC
Industrial wastewater[§]	Manufacture of fruit, vegetable products, and of animal feed from plant products* Manufacture and bottling of soft drinks* Milk, meat and fish-processing industry* Breweries, production of alcohol and alcoholic beverages, malt-houses* Manufacture of gelatin and of glue from hides, skin and bones*		
	Energy, mineral, metal and chemistry industries Waste management Production of pulp from timber or other fibrous materials, paper and cardboard[#] Pre-treatment or dyeing of fibers or textiles[#] Tanning of hides and skins[#] Slaughterhouses and installations for the disposal or recycling of animal carcasses and animal waste[#] Production of food products from animal raw materials (other than milk)[#] and of vegetable raw materials[#] Treatment and processing of milk[#] Surface treatment using organic solvents[#] Production of carbon (hard-burnt coal) or electrographite by means of incineration or graphitization	for each substance emitted, as defined in The Asbestos Directive (87/217/EEC) The Mercury Discharges Directive (82/176/EEC) The Cadmium Discharges Directive (83/513/EEC) The Mercury Directive (84/156/EEC) The Hexachlorocyclohexane Discharges Directive (84/491/EEC) The Dangerous Substance Discharges Directive (86/280/EEC) The Incineration of Waste Directive (2000/76/EC) The Titanium Dioxide Directive (92/112/EEC) Emissions from Large Combustion Plants (2001/80/EC) Discharge of Dangerous Substances into the Aquatic Environment of the Community (2006/11/EC) Waste (2006/12/EC), Hazardous waste (91/689/EEC) and Waste Oils (75/439/EEC)	Directive 2008/1/EC

Type of wastewater	Origin	Requirements for discharges	Legislation
Agricultural wastewater*	Run-off and seepage into the groundwater and surface water of liquids containing livestock manures Effluents from stored plant materials such as silage	no parameters specified Farmers must voluntarily observe a code of good agricultural practices regarding land application of fertilizer, storage of livestock manures and others (Annex II, Directive 91/676) in order to avoid contamination of waters by nitrates. The amount of livestock manure applied to the land each year, including by the animals themselves, shall not exceed 170 kg of nitrogen.	Directive 91/676/EEC
all types of wastewater	any origin	for each substance emitted, as defined in The Mercury Discharges Directive (82/176/EEC) The Cadmium Discharges Directive (83/513/EEC) The Mercury Directive (84/156/EEC) The Hexachlorocyclohexane Discharges Directive (84/491/EEC) The Dangerous Substance Discharges Directive (86/280/EEC)	Directive 2000/60/EC

[§]excluding wastewater originated from installations or parts of installations used for research, development and testing of new products; [*]below the amounts defined in Directive 2008/1/EC, Annex I; [#]for the amounts defined in Directive 2008/1/EC, Annex I; **BOD_5**, Biochemical oxygen demand, before and after five-day incubation; **COD**, Chemical oxygen demand; **TTS**, Total suspended solids; [a] >10 000 p.e.; [b] 10 000 -100 000 p.e. [c] >100 000 p.e., where **1 p.e.** (population equivalent) means the organic biodegradable load having a five-day biochemical oxygen demand of 60 g of oxygen per day; [d] defined as “natural freshwater lakes, other freshwater bodies, estuaries and coastal waters which are found to be eutrophic or which in the near future may become eutrophic if protective action is not taken” (Directive 91/271/EEC).

2.1 Wastewater contamination with antibiotic residues

Over the last decades there has been an increasing interest on the occurrence of antibiotic residues in wastewater, as it has been argued that their presence may contribute to the increase of bacterial antibiotic resistance (Kümmerer 2009a; Kümmerer 2009b; Segura et al. 2009; Rizzo et al. 2013; Berendonk et al. 2015; Berglund et al. 2015). Antibiotics reach urban wastewaters after being excreted by ambulatory and hospitals patients, and by animals undergoing antibiotic therapy (Kümmerer & Henninger 2003). Wastewater treatment plants are, therefore, major portals for the introduction of antibiotics into the aquatic environment, since a significant part of the contaminants present in sewage will not be removed during treatment (Ternes et al. 2004; Michael et al. 2013; Novo et al. 2013). Antibiotics such as macrolides (azithromycin, chlarithromicin), sulfonamides (sulfasalazine, sulfamethoxazole), lincosamides (clindamycine) and trimethoprim have been reported in similar concentrations in raw inflow and treated effluent of two urban wastewater treatment plants (activated sludge), which suggests very poor removal rates for these substances (Birošová et al. 2014). The application of advanced tertiary treatment processes seems to be effective in the removal of some antibiotic residues (Michael et al. 2013). However, the contamination of the environment with antibiotic residues due to inefficient wastewater treatment is still a pressing problem in many countries (Segura et al. 2009; Segura et al. 2015), where state of the art facilities for wastewater treatment cannot be afforded. Again, the discharge of untreated hospital wastewater into municipal collectors is an issue of concern since in these effluents the concentrations of antibiotics can be higher than normally detected in domestic wastewaters (Brown et al. 2006; Segura et al. 2009).

The prevalence of antibiotics in domestic sewage varies according to each substance, as it depends on factors such as their degradation in the animal or human body, biodegradation by environmental microbiota, their solubility/partition coefficient and reactivity of the molecule (Ternes et al. 2004; Jjemba 2006; Kümmerer 2009a;). Nevertheless, although it is known that antibiotic residues can undergo several transformations in the environment (Kümmerer 2009a), most studies focusing on the antibiotic contamination of the environment target only the parent compounds, which probably leads to the underestimation of the concentration of active compounds (Brown et al. 2006).

Table 2 – Examples of human diseases outbreaks associated with wastewater

Infective agent	Country	Period	Source	Reference
Legionella spp.	Norway	2005 and 2008	industrial aeration aeration ponds from a biological wastewater treatment plant	Olsen et al. 2010
Legionella pneumophila	France	2003–2004	contaminated aerosols from a wastewater treatment plant	Nguyen et al. 2006
E. coli O157:H7	USA	2006	irrigation systems contaminated with wastewater	Gelting et al. 2015
Salmonella spp.	Morocco	not specified	wastewater-spreading field	Melloul & Hassani 1999
Norovirus, Astrovirus, Rotavirus, Enterovirus, Adenovirus	Finland	2007	drinking water supplies contaminated with treated sewage	Maunula et al. 2009
Klebsiella oxytoca	Spain	2009-2011	horizontal wastewater drainage system inside an hospital	Vergara-López et al. 2013
Cryptosporidium hominis	Wales	2005	drinking water reservoir and catchment area contaminated with wastewater	Chalmers et al. 2010
Cryptosporidium parvum	South Korea	2012	drinking water systems contaminated with leakage from septic tanks	Moon et al. 2013
Coxiella burnetii	The Netherlands	2011	sewage water treatment plants receiving waste water from goat farms	Schets et al. 2013
Campylobacter jejuni	Finland	2000	runoff of surface water originating from animal sources into groundwater wells used for drinking water abstraction	Hanninen et al. 2003
Salmonella typhi, Vibrio cholerae	Chile	1991	irrigation systems contaminated with wastewater	Shuval 1993
E. coli VTEC	Sweden	2005	irrigation systems contaminated with wastewater from a cattle farm	Soderstrom et al. 2008
Several pathogens associated with gastroenteritis, including *Arcobacter* spp., *Campylobacter* spp., *Giardia* spp., *Salmonella enterica* sv. Typhimurium	USA	2004	well water/groundwater contaminated with wastewater from treatment facilities and septic tanks following extreme precipitation events	Fong et al. 2007 and O'Reilly et al. 2007

2.2 Potential bacteriological risks associated with wastewater

Another risk associated with wastewater seems to be the presence of harmful bacteria from the human or animal gut that are discharged into water collectors via domestic, hospital or industrial effluents. Wastewater may contain microorganisms that are directly (e.g. pathogens) or indirectly (e.g. carriers of genetic determinants of antibiotic resistance), associated with human and animal pathologies, not only because they are the cause of diseases such as gastroenteritis, dysentery and salmonellosis, but because they have a role in the development of resistance to antibiotic therapy (Arthurson 2008; Calhau et al. 2015) (Table 2). There are many studies that document the wide potential for dissemination of microorganisms that enter the environment as a result of wastewater discharge, as well as the numerous pathways through which this can occur. For example, Slekovec and colleagues (2012) were able to document the presence of closely related bacterial lineages (determined based on Pulse Field Gel Electrophoresis and Multi-Locus Sequence typing) of antibiotic resistant *Pseudomonas aeruginosa* in hospital effluent and downstream in the urban water network, both in the receiving wastewater treatment plant and in the river that receives its treated effluent. Similarly, in a study by Vredenburg et al. (2014), it is reported that *E. coli* lineages of clinical relevance isolated from wastewater are also found in urban streams and in gulls across several countries, hinting at the potential for dissemination of bacteria by animals that contact with wastewater. The reuse of wastewater for irrigation may also contribute to the dissemination of antibiotic resistant bacteria and respective resistance genes, being the most dramatic hypothetic consequence the opportunity to enter the human food chain (Varela & Manaia 2013). The presence of fecal indicators in fresh produce irrigated with treated wastewater, although within acceptable levels of risk, has been documented by Cirelli et al. (2012) and Forslund et al. (2010). Long term irrigation of soils with wastewater for agricultural purposes may also result in soil deterioration with increasing risk for contaminant accumulation (Becerra-Castro et al. 2015). This is illustrated by studies such as Dalkmann et al. (2012) where the authors report the accumulation of antibiotics, and also an increase in the microbial biomass and levels of antibiotic resistance genes in agricultural soils subject to long term practices of irrigation with wastewater, in comparison with rain-fed soils.

The use of nutrient-rich activated sludge from wastewater treatment plants as fertilizer is also a potential route for the dissemination of wastewater bacteria, as sludge contains numerous microorganisms carried by wastewater (Arthurson 2008). As reported by Rahube et al. (2014), and similarly to what has been described for wastewater-irrigated

soils, both soil amendment with activated sludge and vegetables grown in such soil contain bacteria and antibiotic resistance genes that are not found in un-amended soils.

2.3 Evolution of bacterial antibiotic resistance in the antibiotic era

The natural molecules we nowadays designate as antibiotics have since long been produced by microorganisms to serve functions as diverse as antagonistic activity, intercellular communication or regulation of gene expression (Yim et al. 2006). The discovery of antibiotics and their application to the clinical practice was one of the major changes in medicine in the 20th century, decreasing morbidity and mortality rates in all age segments. Some of the antibiotics used to inhibit bacteria, such as chloramphenicol, kanamycin, erythromycin and tetracycline, were originally extracted from environmental isolates, such as soil indigenous actinomycetes (Chan et al. 2013). Noticeably, antibiotic producing microorganisms are also frequently antibiotic resistant in order to tolerate the presence of the molecules they synthesize (Hopwood 2007; Allen et al. 2010). It has been argued that the mechanisms of antibiotic resistance have coevolved with the production of antibiotics as a part of the systems these molecules integrate (Aminov 2009), but also filling other functions such as metabolic processes for cell sustenance (Dantas et al. 2008) or detoxification (Davies & Davies 2010). This may be the case with the efflux pumps that although associated with antibiotic resistance, are often unspecific and can export different types of antibiotics as a way of increasing the tolerance of the cell to adverse environments (Nies 2003; Martínez et al. 2009).

Naturally occurring antibiotic resistance is common in the environment, constituting potentially the main and most complex pool of resistance genes, designated as the environmental resistome (Riesenfeld et al. 2004; D'Costa et al. 2007). Indeed, the origin of many antibiotic resistance genes that are reported nowadays in clinical settings can be traced back to genomes of environmental bacteria. For example, genes coding for resistance to aminoglycosides (*armA*, *rtmB*), to cephalosporins (bla_{CTX}) and even for the synthetic quinolones (*qnr* and *qepA*) are originally from soil bacteria (Cantón 2009). The $bla_{\text{CTX-M}}$ enzymes, in particular, which are now globally disseminated among clinical enterobacteria isolates and increasingly reported among the community (Woerther et al. 2013), can be traced back to the chromosomal beta-lactamase KLUC-1 from the members of the genus *Kluyvera* (Cantón et al. 2012). Hypothetically, the human microbiome may also constitute a reservoir of antibiotic resistance genes that can be transferred to human-infecting pathogens (Sommer et al. 2010). In fact, neither the existence of antibiotic

resistance genes (D'Costa et al. 2011) nor their association with mobile genetic elements, which is considered to be the basis for the rise in acquired antibiotic resistance levels observed today (Barlow & Hall 2002), are new phenomena. With such a diverse pool of antibiotic resistance genes and associated mobile genetic elements available, it is hypothesized that the selective pressure constituted by the overuse of antibiotics in clinical and veterinary practice, as animal growth promoters and as pest control agents in agriculture, readily resulted in the emergence and proliferation of populations of antibiotic resistant bacteria (Davies & Davies 2010). The hypothesis of the existence of a causal effect between the rise in the utilization of antibiotics and the emergence of higher levels of antibiotic resistance (reflected both in the increase of the levels of prevalence of resistance and of the minimum antibiotic concentration needed to inhibit bacterial growth) has gained convincing evidences over the years (Goossens 2009; Oleastro et al. 2011; Datta et al. 2012; Bhattacharya et al. 2015). In summary, the use of antibiotics may select positively for bacteria harboring resistance determinants and therefore favor the proliferation of resistant subpopulations without the competition of their antibiotic-susceptible counterparts (Levy & Marshall 2004; Davies & Davies 2010; Sommer & Dantas 2011).

Nowadays, resistance to one or more classes of antibiotics can be found in most known human pathogenic bacteria and in many human commensals (Levy & Marshall 2004; World Health Organization 2014). The list of known antibiotic resistance genes is extensive, as illustrated by the example of the public Antibiotic Resistance Genes Database (ARDB), which alone has more than 20 000 entries (Liu & Pop 2009). For this reason, the World Health Organization considered antibiotic resistance an issue of major global health concern (World Health Organization 2014).

Several bacterial lineages have, by now, become internationally recognized for their potential for multidrug resistance associated with virulence phenotypes. A well-known example is *E. coli* ST131, an emerging lineage of *E. coli* that includes extraintestinal pathogenic (ExPEC) strains from the phylogroups B2, known for its virulence profile and the potential to harbor beta-lactamase enzymes (Oteo et al. 2010; Nicolas-Chanoine et al. 2014). This lineage, besides being widely disseminated in the clinical settings (e.g. Johnson et al. 2010; Novais et al. 2012;), is also commonly isolated from the environment (Dhanji et al. 2011; Vignaroli et al. 2013; Vredenburg et al. 2014; Calhau et al., 2015). The dissemination of methicilin resistant *Staphylococcus aureus* (MRSA) is another classic example of bacteria that has become clinically relevant due to the acquisition of antibiotic

resistance (Appelbaum 2007). MRSA dissemination is linked to the spread of specific lineages (e.g. CC5, CC8, CC22) associated with enterotoxin production, virulence and antibiotic resistance, and which are nowadays considered pandemic (Monecke et al. 2011). Other less-virulent MRSA lineages have become community-associated and although they are commonly found in asymptomatic hosts, their lethal effect as opportunistic pathogens is documented (Monecke et al. 2011; McAdam et al. 2012).

Besides the obvious implications for human health, the dissemination of antibiotic resistant bacteria and antibiotic resistance genes in the environment has the additional problem of creating hotspots of resistance, with conditions that facilitate the emergence of multidrug resistance bacteria (Rizzo et al. 2013). This is in part due to the existence of environmental bacteria that are highly prone to acquire antibiotic resistance genes, constituting privileged reservoirs of these genetic elements, which can then be passed on to other bacteria. This is for example the case with the genus *Aeromonas*, mainly inhabitants of aquatic environments, whose members have been isolated from wastewater exhibiting a multidrug resistant phenotype and harboring plasmids and integrons carrying antibiotic resistance genes that are commonly associated with antibiotic resistance in clinical settings (Moura et al. 2007; Piotrowska & Popowska 2014).

2.4 Common genetic mechanisms behind acquired antibiotic resistance

Although multidrug resistance phenotypes have existed in nature even before the load of anthropogenic pressure found nowadays, this characteristic of bacteria has gained visibility only in the antibiotic era, as it has become an obstacle to the successful treatment of diseases. The classic way in which antibiotic resistance was thought to appear was, initially, through the emergence of resistant clones that would be selected in the presence of the antibiotic (Martínez & Baquero 2000; Blázquez et al. 2012). More recent evidences, however, suggest that the potential of bacteria to acquire foreign DNA through horizontal gene transfer is on the basis of the quick antibiotic resistance dissemination seen in the last decades. Prokaryotes overcome the limitation of waiting for favorable mutations that are selected by the environment due to the existence of processes of horizontal gene transfer, as virtually all resistance genes can be transferred between different taxonomic groups (Walsh 2006; Stokes & Gillings 2011). Bacteria may acquire new genetic elements either by the uptake of naked DNA from the environment (transformation), by active transfer of mobile genetic elements such as plasmids and integrative conjugative elements from other

bacteria (conjugation), or even by bacteriophage-mediated transfer (transduction) (Frost et al. 2005).

The presence of antibiotics may trigger an SOS response that contributes to accelerate the transfer of antibiotic resistance determinants and, consequently, the acquisition of resistance traits. For example, in *Vibrio cholerae,* the presence of ciprofloxacin may induce an SOS response that increases the frequency of transfer of integrative conjugative elements containing determinants of resistance to chloramphenicol, sulphamethoxazole, trimethoprim and streptomycin (Beaber et al. 2004). Also in *V. cholerae* and in *E. coli*, the expression of the integrase gene and the recombination rate of integrons (which may carry a variety of antibiotic resistance gene cassettes) has been shown to increase as a consequence of exposure to mitomycin, ciprofloxacin or trimethoprim (Guerin et al. 2009). At the cellular level, the presence of antibiotics may elicit different types of response, including gene overexpression or gene duplication, all contributing to cope with the stress imposed by antibiotics (Blázquez et al. 2012; Andersson & Hughes 2014). For example, as the biocidal effect of several antibiotics includes the generation of reactive oxygen species (e.g. gentamicin, ampicilin and norfloxacin), it is likely that cellular oxidative stress-protective mechanisms which counteract such effects end up contributing to the emergence of antibiotic resistance (Dwyer et al. 2014). Duplication and overexpression of genes related to antibiotic resistance may also be a consequence of exposure to antibiotics, as demonstrated in *Pseudomonas aeruginosa* exposed to sub-inhibitory concentrations of ciprofloxacin (Brazas & Hancock 2005).

Mutation rates have also been found to be affected by the presence of antibiotics at sublethal concentrations. While high concentrations of a given antibiotic may eliminate most susceptible bacteria, lower concentrations do not kill the bacterial population but may trigger cellular responses that include an increased mutation rate (Kohanski et al. 2010; Andersson & Hughes 2014) and favor the emergence of resistance. This has been demonstrated *in vivo* by Gullberg et al. (2011), who documented the emergence of *de novo* mutants of *E. coli* and *Salmonella enterica* sv. Typhimurium with resistance to tetracyclines, fluoroquinolones and aminoglycosides, in the presence of sub-inhibitory concentrations of the respective antibiotics.

The presence of phenotypes and genotypes associated with resistance has also been suggested by several authors to be one of the pre-existing conditions that can favor the emergence of other types of resistance, especially in the presence of antibiotics. This

phenomenon has been documented in clinical settings, in the course of antibiotic therapeutics, by Toro et al. (2010). The authors were able to match a quinolone susceptible *Salmonella enterica* strain recovered before the administration of the antibiotic therapy and carrying the low-level quinolone resistance gene *qnrS1,* with its putative quinolone resistant counterpart (based on a clonally strong similarity established by Pulse Field Gel Electrophoresis and Multilocus Sequence typing) recovered after the treatment with ciprofloxacin (Toro et al. 2010). The post-ciprofloxacin treatment isolate was found to carry the high-level quinolone resistance associated Ser83>Tyr mutation in the *gyrA* gene, and to have gained resistant to a range of quinolones as well as to several aminoglycosides and to trimethoprim. In *E. coli*, mutation frequencies have been shown to be significantly higher ($\alpha<0.05$) in strains that harbor extended-spectrum beta-lactamases (ESBL) (Baquero et al. 2005), which may favor the appearance of additional resistance mechanisms that are based on the mutation of pre-existing genes, such as is the case with resistance to fluoroquinolones (Bagel et al. 1999).

The weight of co-selection is another factor to be taken into account in the evolution of antibiotic resistance. If genetic determinants of resistance to antibiotics and other substances toxic to the cell are present in the same mobile genetic elements, the selective pressure that favors those surviving to the toxic compounds will result in a co-selection that also favors the presence of antibiotic resistance genes and their dissemination in the population. This is evidenced in reports of co-selection of antibiotic resistance genes with others conferring resistance to heavy metals (e.g. Seiler & Berendonk 2012). In this way, the selective pressure of pollution types other than antibiotic residues may favor the fixation of antibiotic resistance genes in bacterial populations.

Although the evolution of resistance has been tracked down in vivo in the clinical settings, it is worth of notice that when antibiotic residues are discharged to the environment, the range of species that contact with these residues is amplified, and so it is the number of candidates that may be selected for resistance or suffer altered gene expression leading to emergence of antibiotic resistance. Initially it was assumed that the acquisition of antibiotic resistance determinants would decrease the fitness of the host microorganism, *i.e.* its capacity to survive and proliferate in the community, and this hypothesis was supported by the fact that, under laboratory conditions, antibiotic resistance mutants were unstable and short lived in the absence of selective pressure (Andersson & Hughes 2010). However, this may be not a rule for all bacteria. For example, Sandegren et al. (2012) reported similar growth rates in an *E. coli* strain and its antibiotic resistant

plasmid-carrying counterpart when both were grown in the absence of selective pressure. This suggests that strains with acquired antibiotic resistance may not be outcompeted by the antibiotic susceptible fraction of the population once the selective pressure of antibiotics is removed from the environment.

Additionally, the presence of compensatory mutations that restore the fitness after the emergence of antibiotic resistance-related mutations, or genetic linkage responsible for co-selection with other genetic determinants may also contribute to stabilize he presence of antibiotic resistance determinants in a population (Andersson & Hughes 2010). Moreover, with the present levels and dissemination of antibiotic usage, the occurrence of antibiotic resistance determinants in a population is not so much an accessory trait that can be lost in the absence of selective pressure, as it is a trait necessary for survival in general (Davies & Davies 2010).

2.5 The role of wastewater in the emergence and dissemination of antibiotic resistance

Among the many contaminants that are disseminated by wastewater and may pose a health risk are antibiotic resistant bacteria and their genetic determinants (Table 3). Although wastewater treatment effectively decreases the total number of bacterial cells in wastewater, several studies report that a corresponding reduction is not always verified for antibiotic resistant bacteria (Ferreira da Silva et al. 2006; Ferreira da Silva et al. 2007; Novo & Manaia 2010; Vaz-Moreira et al. 2014). Even wastewater that undergoes tertiary treatment is bound to contribute for the dissemination of antibiotic resistance genes, as complete removal of resistance determinants seems unattainable with the wastewater treatment technologies available (e.g. Guardabassi et al. 2002; Lapara et al. 2011). For example, Lapara et al. (2011) detected the tetracycline resistance-associated genes *tet(A), tet(X), tet(W)* and the class 1 integrase gene *intI1* in a wastewater treatment plant effluent that had undergone state-of-the-art advanced tertiary treatment consisting of filtration through a mixed anthracite coal, silica sand, and garnet filter followed by disinfection with sodium hypochlorite, as well as in several locations downstream of the discharge point. Remarkably, wastewater treatment may even be associated with the increase with prevalence of bacteria resistant to some antibiotics. For example, in Novo et al. (2013), significantly ($\alpha<0.05$) higher levels of prevalence of amoxicillin, ciprofloxacin and sulfamethoxazole resistant enterobacteria were detected in treated effluent when compared

to untreated wastewater. The recent application of molecular and culture-independent approaches to the study or antibiotic resistance dissemination allowed for a more detailed perspective of this phenomenon. The boosting effect of the wastewater treatment on resistance prevalence has been reported by Alexander et al. (2015) for the beta-lactamase genes *ampC*, bla_{VIM} and the vancomycin resistance gene *vanA*; by Narciso-da-Rocha et al. (2014) for the multiple resistance regulator *marA*; and by Czekalski et al. (2012) for the sulfonamide resistance genes *sul1* and *sul2*.

The inability of wastewater treatment to completely remove antibiotic resistant bacteria, including of clinical origin, has been widely reported. Examples of this include the isolation of the globally disseminated uropathogenic ST131(O25) *E. coli* from a municipal wastewater treatment plant in the Czech Republic (Dolejska et al. 2011), and of vancomycin-resistant enterococci (VRE) from the treated effluent (Varela et al. 2013) or from the receiving river (Novais et al. 2005) of an hospital effluent-receiving wastewater treatment plant in Portugal. The failure of wastewater treatment to remove genetic determinants of resistance, and their consequent dissemination into the environment, is also evidenced by several studies that document how antibiotic resistance genes initially detected in clinical isolates can be found in wastewater habitats shortly after they have been first reported (Szczepanowski et al. 2008; Rizzo et al. 2013). It has been suggested by several authors (e.g. Reinthaler et al. 2003; Jury et al. 2011) that the dissemination of antibiotic resistance among bacterial populations may actually be favored by the process of sludge treatment due to the special circumstances created in the course of the treatment process. This hypothesis is based on the fact that the dissemination of antibiotic resistance determinants is associated with mobile genetic elements, and that wastewater brings together high densities of these determinants, originated both from the bacteria from clinical settings and from the environment (Szczepanowski et al. 2008; Moura et al. 2010; Koczura et al. 2013; Wang et al. 2013; Biswal et al. 2014).

2.6 The study of antibiotic resistance in wastewater

While the guidelines from the World Health Organization regarding wastewater include the need to monitor the levels of bacteria associated with pathogenicity (World Health Organization 2015), monitoring of antibiotic resistance in aquatic environments and in wastewater is still not contemplated.

Table 3 - Examples of clinically-relevant antibiotic resistance genes detected in raw and in treated wastewater (adapted from Varela & Manaia 2013).

Class	Mechanism type[a]	Examples of gene type	Approach[b]	World region	References
Aminoglycosides	Drug modification	*aac*, *aac6'-aph2''*, *aad*, *aph*, *sat*, *str*	CD/CI-Plasmid	Africa, Asia, Europe	Ferreira da Silva et al. 2007; Li et al. 2009; Szczepanowski et al. 2009; Araújo et al. 2010; Okoh & Igbinosa 2010; Xia et al. 2010; Figueira et al. 2012; Moura et al. 2012
Amphenicols	Drug efflux	*cml*, *cmx*, *flo*	CD/CI-Plasmid	Africa, Europe	Szczepanowski et al. 2009; Okoh & Igbinosa 2010
	Drug modification	*cat*	CD/CI-Plasmid	Asia, Europe	Szczepanowski et al. 2009; Xia et al. 2010; Moura et al. 2012
Beta-lactam	Drug modification	Class A: $bla_{(CTX, GES, NPS, PER, SHV, TEM, TLA)}$; Class B: $bla_{(IMP, VIM)}$; Class C: $bla_{(CMY)}$, *amp*C; Class D: $bla_{(OXA)}$	CD/CI/CI-Plasmid/ Phage	Asia, Europe, North America	Bockelmann et al. 2009; Lachmayr et al. 2009; Li et al. 2009; Szczepanowski et al. 2009; Moura et al. 2012; Xia et al. 2010; Colomer-Lluch et al. 2011
	Target protection	*mec*	CD/CI/ Phage	Australia, Europe	Bockelmann et al. 2009; Börjesson et al. 2009; Barker-Reid et al. 2010; Colomer-Lluch et al. 2011
Glycopeptide	Target modification	*van*	CD	Europe, Middle East	Gajan et al. 2008; Araújo et al. 2010
Macrolides	Drug efflux	*mel*	CD/CI-Plasmid	Europe	Szczepanowski et al. 2009
	Drug modification	*ere*, *mph*	CD/CD-Plasmid/ /CI-Plasmid	Europe	Szczepanowski et al. 2007; Szczepanowski et al. 2009
	Target protection	*erm*	CD/CI/ CI-Plasmid	Europe	Bockelmann et al. 2009; Faria et al. 2009; Szczepanowski et al. 2009; Araújo et al., 2010
Quinolones	Drug Modification	*aac(6')-Ib-cr*	CD	Europe	Figueira et al. 2012
	Target protection	*qnr*	CD/CI-Plasmid	Asia, Europe	Szczepanowski et al. 2007; Szczepanowski et al. 2009; Xia et al. 2010

Class	Mechanism type[a]	Examples of gene type	Approach[b]	World region	References
Rifampicin	Drug modification	*arr*	CD/CI-Plasmid	Europe	Szczepanowski et al. 2009; Moura et al. 2012
Sulfonamides	Target modification	*sul*	CD/CI-Plasmid	Africa, Asia, Europe, Australia	Szczepanowski et al. 2009; Barker-Reid et al. 2010; Okoh & Igbinosa 2010; Xia et al. 2010; Czekalski et al. 2012
Tetracyclines	Drug efflux	*tet*(A, B, C, D, E, G, H, J, K, L, Y, Z, (30), (39), (AP))	CD/CI/ CI-Plasmid	Africa, Asia, Europe, North America	Auerbach et al. 2007; Szczepanowski et al. 2009; Börjesson et al. 2010; Li et al. 2010; Lapara et al. 2011
	Drug modification	*tet(X)*	CD/CI/ CI-Plasmid	Europe, North America	Szczepanowski et al. 2009; LaPara et al. 2011
	Target protection	*tet*(M, O, Q, S, T, W, BP, (36))	CD/CI/ CI-Plasmid	Asia, Europe, North America	Auerbach et al. 2007; Bockelmann et al. 2009; Szczepanowski et al. 2009; Araújo et al. 2010; Li et al. 2010; LaPara et al. 2011
	Other	*tet*(R31, U)	CD/ CI-Plasmid	Europe	Szczepanowski et al. 2009
Trimethoprim	Drug modification	*dfr*, *dhfr*	CD/CI-Plasmid	Africa, Europe	Ferreira da Silva et al. 2007; Szczepanowski et al. 2009; Okoh & Igbinosa 2010; Figueira et al. 2012; Moura et al. 2012
Multidrug	Drug efflux	*acr*, *mex*, *qac*	CD/CI-Plasmid	Asia, Europe	Szczepanowski et al. 2009; Xia et al. 2010

a, DM, drug modification; DE, drug efflux; TM, target modification; TP, target protection (Liu & Pop 2009)

b, CD, culture dependent method; CI, culture independent screening in total or plasmid DNA;

However, the recommendations for the surveillance of antibiotic resistance in the clinical practice (World Health Organization 2014) include several species, such as *E. coli* and *Klebsiella pneumoniae*, that are common inhabitants of wastewater environments and known for their potential as antibiotic resistance carriers (Figueira et al. 2012; Vaz-Moreira et al. 2015). Despite this fact, legislation in Europe has yet to set standards for levels of antibiotic resistance in water destined for human consumption (Council Directive 98/83/EC 1998). In the absence of standardized methods for the monitoring of antibiotic resistance in environmental samples, research has adapted the methods and criteria from the clinical practice (Kahlmeter 2014), including the use culture-dependent methods such as membrane-filtration, enumeration and isolation in antibiotic-supplemented media (Schwartz et al. 2003; Novo & Manaia 2010; Kwak et al. 2014), which allows the comparison of environmental and clinical data. However, since less than 99% of a bacterial community may be culturable (Amann et al. 1995) the development of methods that target the non-culturable populations are necessary to expand the study of antibiotic resistance emergence and dissemination. Despite the usefulness of these methods, the World Health Organization alerts for the importance of determining resistance phenotypes in the clinical practice in order to establish a rapid therapeutic response (World Health Organization 2011). When applied to the study of environmental bacterial populations, culture-independent methods have been used as a complement to culture-dependent techniques, mostly serving the purpose of revealing the richness of wastewater microbial populations. The use of culture-independent methods, based on the analysis of genetic material sampled directly from the environment, has further complemented the study of wastewater bacterial populations. These techniques include the characterization of the bacterial communities based on the analysis of the gene 16S rRNA, the quantification of target genes based on quantitative PCR or the screening of the genetic material based on non-targeted analyses, designated as metagenomics analysis. Analysis of Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis (PCR-DGGE) is a culture-independent method that has proved a useful tool for the study of microbial populations (e.g. Moura et al. 2009). This approach has allowed the study of variations in bacterial populations in different wastewater treatment systems (Adrados et al. 2014), in the course of a specific interval of time (Novo et al. 2013) or the presence of specific conditions such as the presence of antibiotics (Novo et al. 2013; Aydin et al. 2015). The use of PCR-DGGE has been shown to complement the data from both culture dependent methods (Novo et al. 2013) and high throughput sequencing tools such as 454-pyrosequencing (Vaz-Moreira et al. 2011),

contributing to a more comprehensive knowledge of the ecology of microbial populations. Examples of the first metagenomics analyses of antibiotic resistance genes in the environment are the works of Szczepanowski et al. (2008) and Schlüter et al. (2008), which uncovered the high diversity of plasmid-carried genes of resistance to aminoglycosides, beta-lactams, chloramphenicol, fluoroquinolones, macrolides, rifampicin, tetracyclines, trimethoprim and sulfonamides in wastewater using 454-pyrosequencing technology. Over the last years, these studies have become frequent (e.g. Parsley et al. 2010; Kristiansson et al. 2011; Schmieder & Edwards 2012). A complementary approach based both on classic culture-dependent and state of the art culture-independent methods, coupled with an integrated analysis of data from both environmental and clinical sources, could allow the definition of strategies to control and counteract antibiotic resistance dissemination.

3. Objectives

The present study was designed to characterize hospital effluents regarding its potential for antibiotic resistance dissemination in comparison with municipal wastewater and other environments, aiming at contributing for the possible elaboration of guidelines for the adequate management of this type of effluents.

It was aimed specifically at:

i. characterizing hospital effluent and municipal wastewater regarding the concentration of antibiotic and heavy metal residues and the prevalence of antibiotic resistant bacterial populations;
ii. assessing possible correlations between the concentration of antibiotics and heavy metal residues and the presence of antibiotic resistant bacteria and bacterial community composition in municipal and hospital effluents;
iii. inferring about the routes of dissemination of antibiotic resistance bacteria and antibiotic resistance genes from the hospital effluent into other environments by the use of molecular epidemiology tools and the characterization of antibiotic resistance and mobile genetic elements;
iv. assessing the potential for hospital effluent isolates to act as a reservoir of antibiotic resistance through the study of their ability to transfer mobile genetic elements with antibiotic resistance determinants.

4. Thesis outline

Given the privileged role of hospital effluents as environmental hotspots of antibiotic resistance, and the scarcity of studies on this matter, the present study was planned in order to assess the influence of this type of effluent in the dissemination of bacterial antibiotic resistance into the environment. It is composed of five chapters, corresponding to five articles, of which four are published in peer-reviewed international scientific journals and one is in preparation for submission.

The study is based on a 10 month-long sampling campaign, during which a total of 28 samples from an hospital effluent and from the raw and treated wastewater of the receiving urban wastewater treatment plant were collected and processed to obtain extracts of antibiotic residues and metals (performed at Agência Portuguesa do Ambiente), total DNA extracts and bacterial isolates representative of each type of wastewater. When appropriate, samples from other urban wastewater treatment plant (Ferreira da Silva et al. 2007; Novo et al. 2013), from urban streams (Figueira et al. 2011), wild birds and urban seagulls (Vredenburg et al. 2014) and clinical patients (characterized at National Reference Laboratory of Antimicrobial Resistance and Healthcare Associated Infections, National Institute of Health Dr Ricardo Jorge) were included to allow a more extensive comparison.

The first chapter (Varela, A.R., André, S., Nunes, O.C., Manaia, C.M., 2014, **Insights into the relationship between antimicrobial residues and bacterial populations in a hospital-urban wastewater treatment plant system**. Water Res. 54, pp.327–336) is a comparative analysis of hospital and municipal wastewater, where the prevalence of antibiotic resistance and the correlations between antibiotic and heavy metal residues and total and antibiotic resistance bacterial populations are analyzed.

A deeper characterization of the isolates recovered during the sampling campaign was carried out in the following chapters, through different but complementary perspectives.

Chapter 2 (Varela, A.R., Macedo, G.N., Nunes, O.C., Manaia, C.M., 2015, **Genetic characterization of fluoroquinolone resistant *Escherichia coli* from urban streams and municipal and hospital effluents**. FEMS Microbiol. Ecol. 91, pp.1–34) and Chapter 3 (Varela, A.R., Manageiro, V., Ferreira, E., Guimarães, M.A., da Costa, P.M., Caniça, M., Manaia, C.M., 2015, **Molecular evidence of the close relatedness of clinical, gull and wastewater isolates of quinolone-resistant *Escherichia coli***. J. Glob. Antimicrob. Resist. 3, pp. 286–289) aimed to characterize the dissemination of antibiotic resistance by

members of the species *E. coli*, the most important indicator of fecal contamination in water. This species is often the focus of studies concerning wastewater, as it is a classic indicator of the presence of human-associated contamination but also an opportunistic pathogen with ever increasing clinical relevance. Isolates from hospital effluent and the receiving urban wastewater treatment plant, but also from other urban wastewater treatment plants, clinical isolates, urban seagulls and wild birds were characterized with the use of phylogenetic tools, and regarding the presence of antibiotic resistance determinants and antibiotic resistance-associated mobile genetic elements. A subset of these isolates was also tested for their potential for the transmission of antibiotic resistance determinants.

Chapter 4 (Varela, A.R., Nunes, O.C., Manaia, C.M., 2016. **Quinolone resistant *Aeromonas* spp. as carriers and potential tracers of acquired antibiotic resistance in hospital and municipal wastewater**. Sci. Total Environ. 542, pp.665–671) was also based on the characterization of isolates but, in order to obtain a different perspective of the previous chapters, it was focused on a bacterial group that is not in the list of the leading agents of health care associated infections, *Aeromonas* spp. The main objective was to assess the potential of a predominantly environmental bacterial group to act as an antibiotic resistance reservoir, and if possible to infer about routes of dissemination of antibiotic resistance determinants from the clinical context into the environment.

To attain this, a group of *Aeromonas* spp. isolates was identified and genotyped to assess the predominance of different species and genotypes in the different types of wastewater under study. Following a rationale similar to what was applied to the study of *E. coli*, *Aeromonas* spp. isolates were characterized regarding the presence of determinants of acquired antibiotic resistance and of antibiotic resistance-associated mobile genetic elements, and tested for their potential for the transmission of antibiotic resistance determinants.

In Chapter 5 (**Influence of sub-inhibitory concentrations of antibiotics in the transfer rate of the conjugative plasmidome of a hospital effluent multidrug resistant isolate**, submitted for publication), the objective was to get further insights into the transfer of antibiotic resistance determinants from hospital effluent isolates, in order to assess the potential of bacteria from this type of water for antibiotic resistance dissemination. For this, a multidrug resistant isolate which had shown the recurrent ability to transfer both antibiotic resistance determinants and plasmid replicons during conjugation was chosen among the *E. coli* group characterized in Chapters 2 and 3. The conjugation efficiency of this isolate was studied in the presence of sub-inhibitory concentrations of different

antibiotics. The conjugative plasmidome of the isolate, *i.e.*, the plasmidic content transferred by conjugation, was sequenced in order to fully assess the range of antibiotic resistance genes transmitted in the conjugation process, and to infer about a possible explanation for the variations observed in the conjugation frequencies in the presence of sub-inhibitory concentrations of antibiotics.

The results obtained in the course of this study offer an integrated perspective of the potential for hospital effluent to act as a link between the clinical context and the environment and are discussed in the General conclusions.

Chapter 1

Insights into the relationship between antimicrobial residues and bacterial populations in a hospital-urban wastewater treatment plant system

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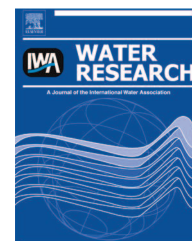

Insights into the relationship between antimicrobial residues and bacterial populations in a hospital-urban wastewater treatment plant system

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ABSTRACT

The relationship between antimicrobial residues, antibiotic resistance prevalence and bacterial community composition in hospital effluent and in the receiving wastewater treatment plant was studied. Samples from hospital effluent, raw inflow and final effluent of the receiving wastewater treatment plant were characterized for amoxicillin and ciprofloxacin resistance prevalence, content of heavy metals and antimicrobial residues and bacterial community structure, based on 16S rRNA gene PCR-DGGE analysis. The concentration of fluoroquinolones, arsenic and mercury was in general higher in hospital effluent than in raw inflow, while the opposite was observed for tetracyclines, sulfonamides and penicillin G. The prevalence of ciprofloxacin resistance was significantly higher in hospital effluent than in raw inflow. The concentration of antimicrobial residues was observed to be significantly correlated with the prevalence of antibiotic resistant bacteria and with variations in the bacterial community. Hospital effluent was confirmed as a relevant, although not unique, source of antimicrobial residues and antibiotic resistant bacteria to the wastewater treatment plant. Moreover, given the high loads of antibiotic residues and antibiotic resistant bacteria that may occur in hospital effluents, these wastewater habitats may represent useful models to study and predict the impact of antibiotic residues on bacterial communities.

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1. Introduction

Over the last decades, bacterial resistance to antibiotics has become an issue of growing concern worldwide (French, 2010), frequently attributed to the excessive use of antibiotics (Kim and Aga, 2007; Martinez, 2009). Antimicrobial residues can impose selective pressures, capable of favoring the proliferation of resistant bacteria, with the progressive elimination of the susceptible organisms. Simultaneously, antimicrobial

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residues may induce bacteria to transfer horizontally antibiotic resistance genes for other community members (Davies and Davies, 2010; Gillings, 2013). It is estimated that antibiotic concentrations measured in environmental samples, such as rivers, can inhibit wild-type bacteria (Tello et al., 2012). Due to such inhibition, antimicrobial residues would be also implicated in the rearrangement of the bacterial communities. This hypothesis was recently supported by the demonstration of significant correlations between the concentrations of antimicrobial residues, antibiotic resistant bacteria or their genes and rearrangements of the bacterial communities in surface and wastewater (Huerta et al., 2013; Novo et al., 2013). In spite of these evidences that could lead to an apparently simple cause–effect relationship, the complexity of propagation of antibiotic resistance in the environment is widely recognized. Intriguing questions are, for example, the effect of sub-inhibitory concentrations of antibiotics on the bacterial communities or on the stability of the resistance phenotypes, even in the absence of selective pressures (Andersson and Hughes, 2010, 2012). The association between the classes of antimicrobial residues found in a given environment and the major types of antibiotic resistance occurring in the same site are also not well understood (Oberlé et al., 2012; Huerta et al., 2013; Novo et al., 2013).

Health care facilities, where the use of antibiotics is more frequent and intensive and where antibiotic resistant bacteria may have a selective advantage over the susceptible counterparts, are regarded as important reservoirs of antibiotic resistance (Kümmerer and Henninger, 2003; Jakobsen et al., 2008; Galvin et al., 2010; Harris et al., 2013; Varela and Manaia, 2013). In addition, in urban areas, wastewater treatment plants represent important receptors for antimicrobial residues and antibiotic resistant bacteria (Manaia et al., 2012; Michael et al., 2013; Rizzo et al., 2013). This situation may be worsened when untreated effluents from health care facilities are received in the urban wastewater treatment plants. Nevertheless, there are no legal requirements for hospital effluents treatment prior to its discharge in the municipal collector. In spite of the potential risks of hospital effluents regarding their role as possible suppliers of antibiotic resistant bacteria to the environment, these effluents represent useful models to assess both the relationship between antibiotic residues and antibiotic resistance and the influence that they may display in the receiving wastewater treatment plant.

The potential effects on microbiome due to pollution with antibiotics have received little attention. However, additional knowledge in this area is fundamental to assess the risks associated with the environmental spread of resistance genes and to control potential adverse effects for human well-being (Gillings, 2013). The current study was based on the hypothesis that hospital effluent is an important supplier of antimicrobial residues and of antibiotic resistant bacteria to the receiving wastewater treatment plant, yielding higher levels of both types of contaminants. Moreover, it was hypothesized that given the different levels of antimicrobial residues and antibiotic resistance observed in hospital and municipal effluents, these wastewater systems would provide interesting insights into the relationship between antimicrobial residues, antibiotic resistant populations and bacterial communities. The assessment of those hypotheses also aimed to infer about the impacts that hospital effluents may have on the occurrence of antimicrobial residues and of antibiotic resistant bacteria in the respective municipal wastewater treatment plant.

2. Materials and methods

This study examined samples from a hospital effluent, and from the raw and treated wastewater of the respective urban treatment plant, located in the northern region of Portugal (Varela and Manaia, 2013). The hospital included in this study serves a population of 3 million people, covering more than 30 clinical specialties and having a capacity of 1120 beds. Over the sampling period, the hospital effluent had an average flow of 1000 m^3/day and average values of Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD) and suspended solids of 622 mg O_2/L, 278 mg O_2/L and 305 mg/L, respectively (source: hospital). The urban wastewater treatment plants, which receives the effluents of this hospital (representing about 0.1% of the daily flow) is prepared to serve a population equivalent of 200,000 inhabitants, and treats domestic sewage (>95%), pre-treated industrial effluents (<2%), untreated effluents from different healthcare facilities (<2%) and storm water. Over the sampling period, it was registered an average flow of 1.1×10^6 m^3/day, and average values of COD, BOD and suspended solids of 699 mg O_2/L, 488 mg O_2/L and 334 mg/L in the raw inflow, and 53 mg O_2/L, 9 mg O_2/L and 19 mg/L in the treated effluent, respectively (source: wastewater treatment plant). In the treatment plant, wastewater undergoes a preliminary treatment where fats and bulky solids are removed. The sewage water is then decanted in a primary settling tank (primary treatment) and further directed to secondary treatment in a biological reactor responsible for the removal of organic matter, composed of an anoxic (denitrification), an aerated (nitrification) and an endogenous (phosphorous removal) zone. The final treatment consists of sand bed filtration, for the removal of suspended solids. Treated effluent is discharged to a river mouth.

2.1. *Sampling*

Seven samples were collected from the hospital effluent (one per month in October 2010, January–April 2011, June–July 2011), 21 from the raw inflow and 21 from the treated effluent of the wastewater treatment plant (three consecutive days in the months of October 2010–March 2011, May 2011) (Fig. S1, S2). Volumes of 5 L of simple samples of hospital effluent or of 24 h composite samples of raw (after the primary settling tank) and treated wastewater (final effluent) of the wastewater treatment plant were collected in glass sterile bottles and in polypropylene flasks, transported refrigerated to the lab and analyzed within 6 h for biological analyses, and 24 h for chemical analyses.

2.2. *Enumeration of cultivable bacteria*

Total and antibiotic resistant bacteria were quantified using the membrane filtration method as described by Novo and Manaia (2010). Briefly, 1–10 mL of decimal serial dilutions

were filtered through cellulose nitrate membranes (0.45 μm pore size, 47 mm diameter, Advantec MFS, Dublin, CA, USA) and were incubated on plate count agar (PCA, Pronadisa, Madrid, Spain) for total heterotrophs (48 h at 30 °C), on m-faecal coliform (mFC, Difco, Maryland, USA) for enterobacteria (48 h at 30 °C), or on glutamate starch phenol-red agar (GSP, Merck, Darmstadt, Germany) which recovers mainly aeromonads/pseudomonads (48 h at 37 °C). Since GSP has poor selectivity against *Pseudomonas* spp. in wastewater samples, incubation was performed at 37 °C, in an attempt to promote the preferential growth of *Aeromonas* spp., given the good capacity of members of this genus to grow at that temperature (Martin-Carnahan and Joseph., 2005). The subpopulations retrieved on the same media supplemented either with 32 mg/L of amoxicillin (AML, Sigma, Steinheim, Germany) or 4 mg/L of ciprofloxacin (CIP, Sigma, Steinheim, Germany) were considered resistant to the respective antibiotic. These concentrations of amoxicillin or ciprofloxacin correspond to the minimal inhibitory concentrations and were assayed in previous studies (Novo and Manaia, 2010; CLSI, 2012; Novo et al., 2013). All procedures were performed in triplicate. On each culture medium, enumeration was performed for plates displaying 10–90 colony forming units (CFU) (Gerhard et al., 1981). The percentage of antibiotic resistance was determined as the ratio between the CFU/mL formed on the culture medium with and without antibiotic.

2.3. Bacterial community characterization by DGGE analysis

Samples filtration, DNA extraction, and 16S rRNA gene based denaturing gradient gel electrophoresis (DGGE) analysis was made as described before (Novo et al., 2013). Briefly, total DNA was extracted in triplicate from 21 samples (seven from the hospital effluent, seven from the raw inflow and seven from the treated effluent, corresponding to the second of the three consecutive days sampled in the wastewater treatment plant), after filtering 150 mL (wastewater treatment plant treated effluent) or 25 mL (hospital effluent and wastewater treatment plant raw inflow) through polycarbonate membranes (0.2 μm porosity, Whatman, Brentford, UK). DNA extraction was performed using the PowerWater DNA Isolation kit (MOBIO Laboratories Inc., Carlsbad, CA, USA), following manufacturer's instructions. The efficiency of DNA extraction was assessed by DNA quantification using fluorometry (QubitTM Fluorometer, Invitrogen, USA). A 180 bp fragment of the 16S rRNA gene, corresponding to the region V3, was amplified using the primers 338F-GC-clamp (5′-GACTCCTACGGGAGGCAGCAG-3′ with a GC clamp) and 518R (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer et al., 1993). PCR reactions (Biometra), quality control and DGGE analysis (DCode™ universal mutation detection system, Bio-Rad Laboratories) were made according to previous description (Novo et al., 2013). Approximately 1.8 μg of DNA were loaded on an 8% (w/v) vertical polyacrylamide gel with a 28%–59% denaturing gradient (where 100% denaturing gradient is 7 M urea and 40% deionized formamide. Reproducibility of the DGGE patterns was assessed by including a reference DNA extract with a known DGGE profile in all gels. Gel staining and image visualization and acquisition (Molecular Imager Gel Doc XR system, Bio-Rad Laboratories) were performed as described previously (Barreiros et al., 2008). DGGE profiles were observed visually and further analysed using the Bionumerics software (version 6.1, Applied Maths), with the profiles comprising of reference organisms serving as standard/normalization lanes for inter-gel comparison. Band classes were attributed according to band position and a two-entry table including band intensity value per band class was generated for each DGGE profile, to allow further comparison of samples.

Bands presenting significant positive correlations with the variation of the concentration of antibiotics, heavy metals or with the percentage of resistant bacteria were selected for identification based on DNA sequencing analysis. In order to detect possible cases of co-migration in a single DGGE band, these bands were excised, amplified, cloned with InsTAaclone™ cloning kit (Thermo Scientific) and analyzed by DGGE as described by Novo et al. (2013). Clone inserts that matched the original band in the DGGE pattern were sequenced with the primer M13F-pUC (5′-GTTTTCCCAGT-CACGAC-3′). Nucleotide sequences were checked manually for their quality and their phylogenetic affiliation was determined using the BLAST software (http://blast.ncbi.nlm.nih.gov/) to query the GenBank database.

2.4. Quantification of micropollutants

Water samples were characterized for their content of the metals cadmium, chromium, lead, arsenic and mercury and the antimicrobials tetracycline, oxytetracycline, doxycycline, chlorotetracycline, penicillin G, penicillin V, sulfamethazine, sulfathiazole, sulfamethoxazole, ciprofloxacin and ofloxacin. Cadmium, chromium and lead content was determined by inductively coupled plasma atomic emission spectroscopy; arsenic content was determined by hydride generation atomic absorption spectroscopy and mercury content was determined by cold vapor atomic absorption spectrometer as described by Novo et al. (2013). Antibiotic content was determined by liquid chromatography coupled with electrospray ionization mass spectrometry, after solid-phase extraction (Novo et al., 2013).

2.5. Statistical analyses

Bacterial counts (logCFU/mL) and antibiotic resistance percentage values (%) were compared using one-way ANOVA and the post-hoc Tukey test (SPSS 19.0 for Windows) and considered significantly different when $p < 0.01$. Variation of the bacterial communities structure and composition and/or the antibiotic resistance abundance were assessed in function of time, type of water, concentration of antimicrobial residues or percentage of antibiotic resistance using Detrended Correspondence or Canonical Correspondence Analyses (DCA and CCA, software package CANOCO version 4.5). For this purpose two-entry tables of DGGE bands intensity, percentage of antibiotic resistance and concentration of antimicrobial residues were prepared. The significance of the relationship between DGGE patterns, percentage of antibiotic resistance and antibiotic/heavy metal residues was tested by Monte Carlo permutations test ($n = 499$). Explanatory variables included in CCA analyses were selected by manual forward selection

including the permutation test (Monte Carlo permutations test) (Novo et al., 2013).

3. Results

3.1. *Quantification of antimicrobial residues*

In general, higher concentrations of ciprofloxacin, ofloxacin, arsenic and mercury were found in the hospital effluent than in the raw inflow (Table 1). In contrast, the concentrations of sulfamethoxazole, tetracycline and penicillin G were lower in the hospital effluent than in the raw inflow. Other antibiotics and metals quantified were, most of the times, below the limit of quantification (LOQ) (Table 1). The concentrations of antibiotics detected varied over time, particularly in the hospital effluent, most probably due to the patterns of utilization and consumption of these antibiotics in the institution (data not available). In the final effluent of the wastewater treatment plant, the concentrations of antibiotics were generally lower than in the raw inflow, although higher inputs corresponded to higher final concentrations. Among the heavy metals examined, in the wastewater treatment plant, mercury was detected above the quantification limit only occasionally and arsenic was always present in concentrations above 2 μg/L. The concentration of arsenic was not reduced after wastewater treatment. The other antibiotics and metals analyzed were found most of the times below the limit of quantification (Table 1).

3.2. *Total and antibiotic resistant cultivable bacterial populations*

The counts of cultivable heterotrophs and aeromonads/pseudomonads in hospital effluent and in raw inflow were not significantly different ($p > 0.01$), ranging 10^6–10^7 CFU/mL. In contrast, the hospital effluent presented significantly lower ($p < 0.01$) counts of enterobacteria than the raw inflow (Fig. S1), differing by one log cycle. Accordingly, hospital effluent presented significantly lower counts of amoxicillin resistant enterobacteria ($p < 0.01$), than the raw inflow. For the other bacterial groups examined, the counts of antibiotic resistant bacteria in hospital effluent and raw inflow were not significantly different and ranged from 10^5 to 10^7 CFU/mL (Fig. S1). In the final effluent of the wastewater treatment plant, the counts of total and antibiotic resistant bacteria were about 1.5–2 logarithmic cycles lower for the three bacterial groups.

The percentage values of amoxicillin and of ciprofloxacin resistant bacteria were compared for the three types of water (Table 2). These two resistance phenotypes revealed different trends. Although higher in hospital effluent than in raw inflow, amoxicillin resistance prevalence did not differ significantly between both sites ($p > 0.01$). In contrast, ciprofloxacin resistance was significantly more prevalent ($p < 0.01$) in the hospital effluent than in the raw inflow, for all the bacterial groups examined. Wastewater treatment did not affect the prevalence of antibiotic resistance, since non-significantly different values were observed between the raw inflow and the treated effluent (Table 2).

3.3. *16S rRNA gene-DGGE characterization of the bacterial community*

The characterization of the bacterial community through 16S-rRNA gene based DGGE analysis allowed the assignment of a total of 26 band classes (Table 3, Fig. S2). Among these, seven bands were present in all samples examined from hospital effluent and raw inflow (B1, B5, B6, B7, B9, B19 and B24). These seven bands were detected in all (B5) or in more than half of the treated effluent samples examined, suggesting that the populations represented by these bands may persist from the hospital to the final effluent of wastewater treatment plant (Table 3, Fig. S2). Band intensity, presumably related with population relative abundance, of the DGGE patterns was compared based on a detrended correspondence analysis (DCA). This analysis led to the distribution of the DGGE profiles over two axes, accounting respectively, for 19.4% and 10.7% of

Table 1 – Concentration of antibiotic residues and heavy metals determined in wastewater over the sampling period.

		Mean (range) values of antibiotic/heavy metal concentration in wastewater samples		
		Hospital effluent ($n = 7$)	Raw inflow ($n = 21$)	Treated effluent ($n = 21$)
Antibiotics (μg/L)	Penicillin G	0.86 (<0.12–1.39)	1.03 (<0.12–2.23)	0.80 (<0.12–1.43)
	Penicillin V	(<0.13–0.63)	(<0.13–0.42)	(<0.13–0.30)
	Ciprofloxacin	0.88 (<0.13–2.53)	0.44 (<0.13–0.86)	0.27 (<0.13–0.43)
	Ofloxacin	0.59 (<0.13–1.42)	0.34 (<0.13–0.73)	0.23 (<0.13–0.30)
	Sulfamethoxazole	0.89 (<0.12–1.54)	0.83 (0.42–3.00)	0.35 (0.16–0.55)
	Sulfathiazole	(<0.12–0.28)	(<0.12–0.17)	(<0.12)
	Sulfamethazine	(<0.13–1.70)	(<0.13–1.30)	(<0.13)
	Tetracyclin	1.06 (0.52–2.14)	1.75 (0.44–4.16)	0.99 (<0.12–2.42)
	Oxytetracycline	(<0.14)	(<0.14)	(<0.14)
	Doxycycline	(<0.12–0.66)	(<0.12–0.18)	(<0.12–0.18)
	Chlortetracycline	(<0.12)	(<0.12–0.83)	(<0.12)
Heavy metals (μg/L)	As	3.68 (2.20–6.50)	3.18 (2.00–4.90)	2.54 (1.90–3.40)
	Cd	(<10)	(<10)	(<10)
	Cr	(<10–14)	(<10–30)	(<10–15)
	Hg	1.17 (<0.05–3.30)	0.29 (<0.05–0.96)	<0.05
	Pb	(<10–20)	(<10)	(<10)

Table 2 – Prevalence of antibiotic resistance prevalence in wastewater over the sampling period.

Bacterial group (Isolated on)	Wastewater samples	Mean (range) values of antibiotic resistance prevalence (%)	
		+32 μg/mL amoxicillin	+4 μg/mL ciprofloxacin
Heterotrophs (PCA)	Hospital effluent ($n = 7$)	37.8a (11.2–77.6)	19.5a (1.6–40.3)
	Raw inflow ($n = 20$[a])	28.3a,b (9.5–68.0)	7.6b (0.6–37.5)
	Treated effluent ($n = 20$[a])	23.7b (1.6–66.3)	9.3b (0.4–80.1)
Aeromonads/Pseudomonads (GSP)	Hospital effluent ($n = 7$)	48.8a (10.3–82.5)	10.7a (1.2–35.2)
	Raw inflow ($n = 20$[a])	41.3a,b (1.5–94.6)	3.7b (0.1–17.6)
	Treated effluent ($n = 20$[a])	29.0b (5.4–72.1)	3.3b (0.2–31.6)
Enterobacteria (mFC)	Hospital effluent ($n = 7$)	52.7a (4.0–91.9)	15.8a (0.6–52.5)
	Raw inflow ($n = 20$[a])	45.7a (0.2–88.5)	3.5b (0.1–14.1)
	Treated effluent ($n = 20$[a])	46.4a (0.1–96.9)	5.1b (<0.1–18.9)

a, b – significantly different ($p < 0.01$) in the three types of water on the basis of post hoc Tukey test for the comparison of samples of different origins.
[a] No data was available for one sample.

the bacterial community variation (Figs. S2 and S3). The DCA biplot allowed the distinction of hospital effluent, raw inflow and treated effluent population patterns. Hospital effluent and raw inflow samples separated from treated effluent over axis 1 and hospital effluent samples separated from wastewater treatment plant samples, mainly along axis 2. Although the bacterial communities from the three types of water could be distinguished by DCA analysis, the DGGE patterns of raw inflow were closer to those of hospital effluent than of treated effluent. The bacterial populations of hospital effluent samples collected in April (4) and June (6) differed from those of other hospital effluent samples, being closer to the raw inflow bacterial populations. In opposition, the separation of treated effluent DGGE profiles from all the others, suggests alteration of the wastewater bacterial community during treatment.

3.4. *Correspondence analysis between antimicrobial residues and the cultivable antibiotic resistant bacteria*

Canonical correspondence analysis (CCA) evidenced significant correlations between the patterns of variation of the concentration of penicillin G, tetracycline, ciprofloxacin and arsenic (Fig. S4A, B) and the percentage of antibiotic resistant bacteria. The variation of the concentration of ciprofloxacin and arsenic was mainly correlated with axis 1, along which were distributed the ciprofloxacin resistant bacterial

Table 3 – Presence, dominance and affiliation of bacterial populations, represented by DGGE bands, which were positively correlated with the concentration of antibiotic residues and heavy metals or the prevalence of antibiotic resistance.

Band	Affiliation	Presence (number of occurrences/number of samples; %) Dominance (number of occurrences with more than 1/2 of the maximum intensity detected/number of samples; %)			Correlated with: concentration/resistance percentage
		Hospital effluent	Raw inflow	Treated effluent	
B1	*Bacteroidetes*, related with the genus *Prevotella* (AB649279.1, 99%; AB244770.1, 98%)	100 57	100 14	57 14	Ciprofloxacin, ofloxacin, arsenic/ciprofloxacin resistance
B7	*Bacteroidetes*, related with the genus *Alistipes* (AB554232.1, 100%) *Proteobacteria*, related with the genus *Acinetobacter* (KF049130.1, 99%)	100 71	100 100	71 29	Sulfamethoxazole, tetracycline
B13	*Firmicutes*, related with the genus *Faecalibacterium* (JN037416.1, 100%)	100 86	57 0	57 0	Ciprofloxacin, ofloxacin, arsenic/ciprofloxacin resistance
B24	*Proteobacteria*, related with the genus *Aeromonas* (KC906261.1, 100%) *Firmicutes*, related with the class *Clostridia* (JN713307.1, 94%)	100 71	100 43	86 0	Ciprofloxacin, ofloxacin, arsenic/ciprofloxacin resistance
B25	*Firmicutes*, related with the class *Clostridia* (NR_102880.1, 100%)	86 14	100 43	86 0	Sulfamethoxazole, tetracycline

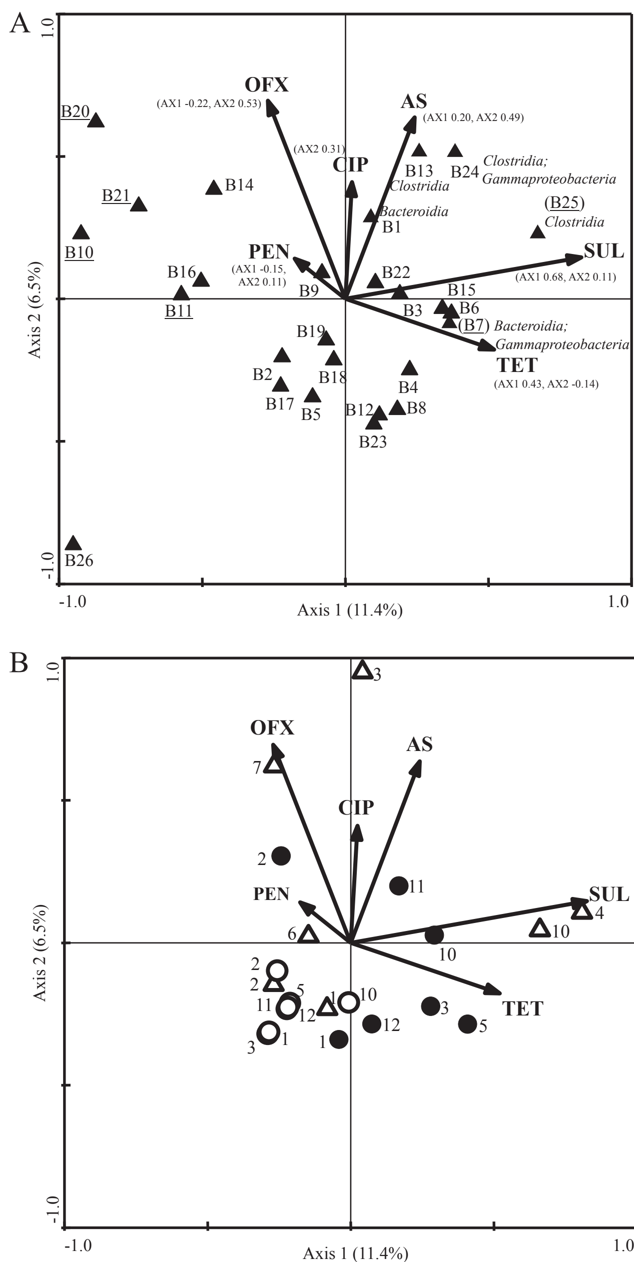

Fig. 1 – Figure 1A. Correspondence Canonical Analysis (CCA) of the variation of 16S rRNA gene-DGGE patterns of hospital effluent, urban wastewater treatment plant raw inflow and treated effluent (the score of variation of the DGGE bands, ▲, was of 0.615, 11.4% over axis 1 and 6.5% over axis 2), in function of the concentration of antimicrobial residues tetracycline (TET, $p = 0.002$), sulfamethoxazole (SUL, $p = 0.002$), ciprofloxacin (CIP, $p = 0.002$), penicillin G (PEN, $p = 0.018$), ofloxacin (OFX, $p = 0.002$) and arsenic (AS, $p = 0.002$). Only the variables significantly ($p < 0.05$) explaining the observed community variation are shown. The species–environmental correlations for axes 1 and 2 were, respectively, 0.829 and 0.765. 16S rRNA gene-DGGE bands with a fraction of variance over axes 1 higher than 0.2/1 are underlined. Figure 1B. Correspondence Canonical Analysis (CCA), as described in Fig. 1A. Samples of hospital effluent (△) and of the raw inflow (●) or treated effluent (○) of wastewater treatment are numbered according to the month of collection (1, January–12, December). Sampling occurred between October 2010 and July 2011.

populations (on PCA, GSP and mFC). Therefore, was evidenced a positive correlation between the variation of the concentration of ciprofloxacin and arsenic and of ciprofloxacin resistance prevalence (Fig. S4A), influenced by hospital effluent samples of March (samples 3 in Fig. S4B). The variation of concentration of penicillin G and tetracycline, distributed over axis 2, was positively correlated with the variation of the percentage of amoxicillin resistant aeromonads/pseudomonads. Given the low weight of axis 2, the observed correlation between the variation of penicillin G and tetracycline concentration and percentage of amoxicillin resistance was weaker than that of arsenic and ciprofloxacin concentration with ciprofloxacin resistance.

3.5. *Correspondence analysis between antimicrobial residues and the bacterial community*

CCA evidenced positive significant correlations ($p < 0.005$) between the variation of the concentration of ofloxacin, penicillin G, ciprofloxacin, sulfamethoxazole, tetracycline, arsenic and the intensity of some DGGE bands (Fig. 1A,B). The variation of the concentration of sulfamethoxazole and tetracycline was mainly correlated with axis 1. The peaks of concentration of these antibiotics in hospital effluent in October and April may explain such a distribution (Fig. 1B, Table 1). Band B7, which comprised bacteria related to the genera *Alistipes* and *Acinetobacter*, and band B25 related to the class *Clostridia* (Table 3, Fig. S2), were those whose variation (in intensity and/or occurrence) presented stronger correlation with the variation of the concentration of those antibiotics. These bands were detected in most hospital effluent and wastewater treatment plant samples. In contrast, the variation of intensity of bands B10, B11, B20 and B21 and of the concentration of sulfamethoxazole and tetracycline was negatively correlated. Most of these bands, although present in hospital effluent and/or raw inflow were more frequent and abundant in treated effluent (Table 3, Fig. S2). Variations in the concentration of fluoroquinolones (ciprofloxacin and ofloxacin) and arsenic were also correlated with changes in the bacterial community composition. The variation of the concentration of fluoroquinolones and arsenic and of populations represented by bands B1, related to the genus *Prevotella*, B13, related with the genus *Faecalibacterium* and B24, related with the genus *Aeromonas* and the class *Clostridia*, was positively correlated. Bands B1 and B24 were more intense or frequent in hospital effluent and raw inflow than in treated effluent and B13 was present and intense in most of hospital effluent samples (Table 3, Fig. S2). The variation of the intensity of band B26, never detected in the hospital effluent, occasionally observed in the raw inflow and always present in the treated effluent, was negatively correlated with the variation of the concentration of fluoroquinolones and arsenic. In general, the results suggest that within the bacterial community, different

populations have variations coincident with fluctuations on the concentration of sulfonamides and tetracyclines or of fluoroquinolones and arsenic.

3.6. Correspondence analysis between cultivable antibiotic resistant bacteria and the bacterial community assessed by DGGE analysis

CCA showed significant correlations between the variation of the percentage of cultivable antibiotic resistant bacteria and DGGE bands intensity (Fig. S5A, B). The variation of the percentage of ciprofloxacin resistant heterotrophs and amoxicillin resistant aeromonads/pseudomonads was mainly distributed over axis 1. The variation of the percentage of enterobacteria (mFC) resistant to ciprofloxacin and of heterotrophs resistant to amoxicillin was distributed over axes 1 and 2 and of amoxicillin resistant enterobacteria over axis 2. The sample of hospital effluent of March was probably influencing this pattern of distribution (sample 3 in Fig. S5B). Bands distributed over axis 1 were those which variation in intensity and/or occurrence was most correlated resistance prevalence. Curiously, the DGGE bands whose variation had the strongest correlation with the variation of ciprofloxaxin resistant bacteria and with the variation of the concentration of fluoroquinolones and arsenic were the same, *i.e.* B1, B13 and B24 (Fig. 1A). In contrast, as observed in the CCA biplot for antimicrobial residues concentrations (Fig. 1A), the variation of band B26, which was never detected in hospital effluent and was always present in treated effluent, was negatively correlated with the variation of the percentage of antibiotic resistant bacteria.

4. Discussion

Because antibiotic consumption is higher and more intense in hospitals than in the community, the development of antibiotic resistant bacteria and the discharge of antimicrobial residues and resistant bacteria in these effluents are supposed to have strong impacts on the spread of antibiotic resistant bacteria in the environment (Kümmerer and Henninger, 2003; Baquero et al., 2008). The combination of three major adaptation mechanisms may contribute to explain the spread and selection of antibiotic resistant populations: i) the occurrence of horizontal transfer of antibiotic resistance genes (Baquero, 2004; Walsh, 2006); ii) the preferential proliferation of resistant bacteria due to selective pressures imposed by antimicrobial residues or heavy metals (Graham et al., 2011; Tello et al., 2012; Hellweger, 2013) and iii) the bacterial community rearrangement to fit the occurrence of substances with antimicrobial activity, such as antimicrobial residues or heavy metals (Lawrence et al., 2008; Graham et al., 2011; Gillings, 2013; Huerta et al., 2013; Novo et al., 2013). Mechanisms ii) and iii) were those supporting our hypotheses and experimental design. In addition, by comparing wastewater habitats supposedly with different levels of contamination with antimicrobial residues and antibiotic resistant bacteria (hospital and municipal wastewater), we intended to get additional insights about the capability of those contaminants to contribute to shape the bacterial communities. However, it should be noted that the aim of the study was to assess the dynamics of the bacterial populations, and if such variations could be correlated with the occurrence of antimicrobial residues. Since such a dynamics may occur regardless the fact that resistance is acquired or intrinsic, no specific discussion on resistance acquisition by horizontal gene transfer or mutation was addressed in this study.

Though antimicrobial residues and antibiotic resistant bacteria may be more abundant in hospital effluents than in the receiving municipal wastewater treatment plant, the dilution of the hospital effluent in the municipal sewage may blur their possible impacts. The comparison of the concentration of antimicrobial residues or prevalence of antibiotic resistance in both hospital effluent and raw inflow is possible contribute to overcome this artifact. This rationale was used to compare the data regarding antimicrobial residues and heavy metals concentrations and antibiotic resistance prevalence. Among the antimicrobial residues and heavy metals analyzed, only ciprofloxacin and ofloxacin, tetracycline, sulfamethoxazole, penicillin G, and the metals arsenic and mercury were detected regularly at levels above the limit of quantification. Confirming our hypothesis, hospital effluent was observed to be an important source of fluoroquinolones and arsenic to the wastewater treatment plant, presenting, in general, higher concentrations than those observed in raw inflow. However, other sources, such as domestic use and other human or veterinary health care facilities, could be responsible for the discharge of sulfamethoxazole, penicillin G and tetracycline to the wastewater treatment plant, since, in general, higher concentrations of these antimicrobials were found in the raw inflow than in the hospital effluent. Regarding antibiotic resistance, hospital effluent presented higher resistance prevalence than the raw inflow or treated effluent for both amoxicillin and ciprofloxacin. However, hospital effluent was not demonstrated as a significant source of amoxicillin resistance. Probably, this was due to the widespread occurrence of intrinsic amoxicillin resistance in environmental bacteria, in particular in *Gammaproteobacteria* (Paterson, 2006; Parker and Shaw, 2011). In contrast, ciprofloxacin resistance was significantly higher (7–10%) in hospital effluent than in raw inflow. A positive correlation between ciprofloxacin resistance and the concentration of that antibiotic and arsenic (Fig. S4A) may suggest a selective pressure effect. Indeed, hospital effluent samples collected in March presented the highest concentration of ciprofloxacin, being also those showing the highest percentage of ciprofloxacin resistance. The observation of significant correlations between the concentration of antimicrobial residues and the prevalence of antibiotic resistant bacteria or resistance genes has been reported in previous studies (Huerta et al., 2013; Novo et al., 2013). Huerta et al. (2013) observed an association between macrolides concentration and the normalized number of copies of the genes *erm*B and *sul*I (resistance determinants to macrolides and sulfonamides, respectively), and a negative correlation between Cd concentration and the abundance of the gene *qnr*S (resistance determinant to quinolones). Novo et al. (2013) observed a significant positive correlation between tetracyclines concentration and the loads of antibiotic resistant bacteria,

although such association was not specifically with tetracycline resistance. As reviewed recently, antibiotic contamination can promote both the mobilization and fixation of resistance genes not only among human and animal commensal bacteria but also between environmental and clinically-relevant species (Baquero et al., 2013; Gillings, 2013). However, major questions are still to be answered. Indeed, the factors that contribute to promote such processes or how these factors interact are not clear yet. The pharmaco-dynamics of the antimicrobial compound as well as the chemical stability or capability to form complexes, and the effect the abiotic conditions such as temperature, pH, or nutrient availability, among others, are probably important elements to clarify the role of these potential selective pressures.

The environmental contamination with antibiotics has non-target effects at gene, cell, and population levels, since antibiotics can trigger different effects such as SOS response, enhance the mutation rate or promote genetic recombination events (Gillings, 2013). For this reason, as Baquero et al. (2013) emphasized recently, a thorough understanding of antibiotic resistance evolution requires a wide vision of the ecology of bacteria. Rearrangements of the aquatic bacterial communities coinciding with variations on the concentration of antibiotic residues were described for urban and industrial pharmacy wastewater or for surface water (Li et al., 2011; Huerta et al., 2013; Novo et al., 2013). As Novo et al. (2013) reported before, also in this study sulfamethoxazole and tetracycline presented a pattern of correlation with the bacterial community distinct of that observed for quinolones. Although the simultaneous measurement of the concentration of antimicrobial agents and of biological indicators (bacterial populations or genes) seems to be an interesting approach to assess possible effects of the pollutants on the microbiota (Graham et al., 2011; Huerta et al., 2013; Novo et al., 2013), it is important to bear in mind the numerous bias that may be involved. In particular, the mobility and half-life of different antimicrobial agents, which are also influenced by the environment where they are discharged, are expected to have a strong influence. For instance, ciprofloxacin is recognized by its high sorption capacity, while tetracyclines, also with high sorption potential, are able to form complexes with calcium or magnesium (Kümmerer, 2009). On the other hand, penicillin G is expected to be rapidly hydrolyzed in the environment due to microbial activity, while sulfonamides, despite the expected elimination by sorption are common contaminants of aquatic environments (Halling-Sørensen et al., 1998; Kümmerer, 2009). Nevertheless, in this study it was suggested that the bacterial populations of the hospital effluent, more than those from raw inflow, can respond to variations of antibiotic concentrations, even when higher concentrations are found in raw inflow. This was observed for tetracyclines and sulfonamides (Fig. 1B). It is thus suggested that hospital effluents can be interesting models to study relationships between antimicrobial residues and bacterial populations.

The observation of significant positive correlations between the variation of the concentration of antimicrobial residues and the presence of some members of the bacterial community may suggest that some of those micropollutants may have a role in shaping the bacterial communities. Variations on the abundance of members of the classes *Gammaproteobacteria*, *Bacteroidia* and *Clostridia*, represented by bands B1, B13 and B24, were correlated with the variation of the concentration of antimicrobial residues. For some of those bacterial groups, not much is known in terms of antibiotic resistance. Nevertheless, it is interesting to note that the same bacterial populations, represented by bands B1, B13 and B24, were also positively correlated with the variation of the percentage of cultivable antibiotic resistant bacteria. These results are in line with the study of Novo et al. (2013), suggesting that some environmental bacteria may be influenced by the occurrence of antimicrobial residues and antibiotic resistant bacteria. In that study, the occurrence of *Epsilonproteobacteria* (related with the genera *Arcobacter* and *Sulfurimonas*) was positively correlated with the concentration of antibiotics. Studies seeking for possible relationships between bacterial community variations and the contamination with antimicrobial residues (Huerta et al., 2013; Novo et al., 2013; the present study) may contribute to assess the potential of antimicrobial agents in disturbing the bacterial communities or in exerting selective pressure effects. As hypothesized in our experimental design, the comparison and multivariate analyses of habitats with different levels of antimicrobial residues and antibiotic resistant bacteria may bring interesting insights into the relationship between antibiotic resistance, antibiotic residues and bacterial populations in water. Hospital effluent was confirmed as an important, although not unique, source of, antimicrobial residues and antibiotic resistant bacteria in wastewater treatment plant. Since there are recognized limitations for the complete removal of antibiotic resistant bacteria and/or antibiotic residues in conventional wastewater treatment systems (Galvin et al., 2010; Czekalski et al., 2012; Chen and Zhang, 2013; Novo et al., 2013), special attention should be given to the discharge of untreated hospital effluents into the municipal collectors.

5. Conclusions

- Ciprofloxacin resistance was significantly more prevalent ($p < 0.01$) in hospital effluent than in raw inflow, for all the bacterial groups studied. In contrast, amoxicillin resistance prevalence was not significantly different in both sites ($p > 0.01$).
- Ciprofloxacin and arsenic concentrations were positively correlated with ciprofloxacin resistance prevalence, mainly in hospital effluent samples.
- The bacterial populations correlated with the concentrations of sulfamethoxazole and tetracycline were different of those correlated with ciprofloxacin, ofloxacin and arsenic;
- A positive correlation was observed between the concentration of sulfamethoxazole and tetracycline and the intensity of the DGGE bands comprising of members of the classes *Bacteroidia* and *Gammaproteobacteria*.
- A positive correlation was observed between the concentrations of ciprofloxacin, ofloxacin and arsenic and the

intensity of the DGGE bands comprising of members of the classes *Clostridia*, *Gammaproteobacteria* and *Bacteroidia*.

- Higher intensities of DGGE bands comprising of the bacterial groups *Bacteroidia* and *Clostridia* were found in samples presenting both higher concentration of fluoroquinolones and arsenic and ciprofloxacin resistant bacteria.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.02.003.

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Appendix A. Supplementary data

The following are the supplementary data related to this article:

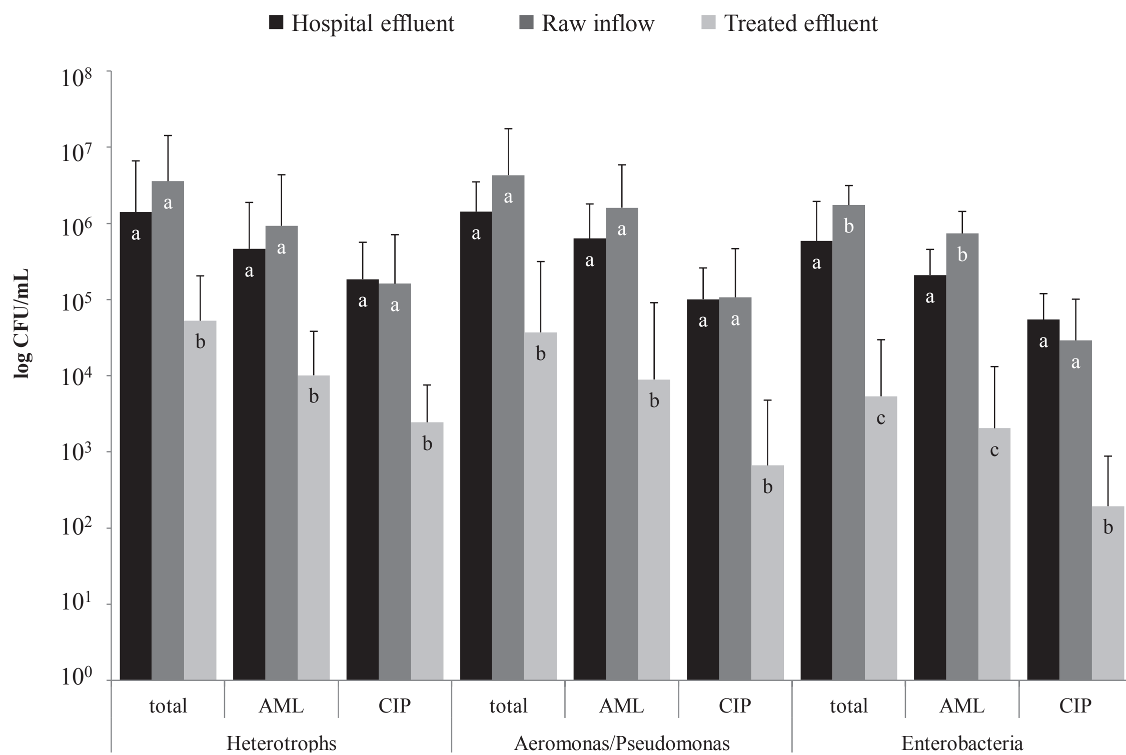

Figure S1. Bacterial counts (colony forming units, CFU/mL) on PCA, GSP and mFC (total), and on these media supplemented with 4 mg/L ciprofloxacin (CIP) or 32 mg/L amoxicillin (AML), in the hospital effluent and in the raw inflow and treated effluent of the respective wastewater treatment plant. a, b, c – homogeneous subsets on the basis of Tukey test of $\log_{10}$(CFU/mL).

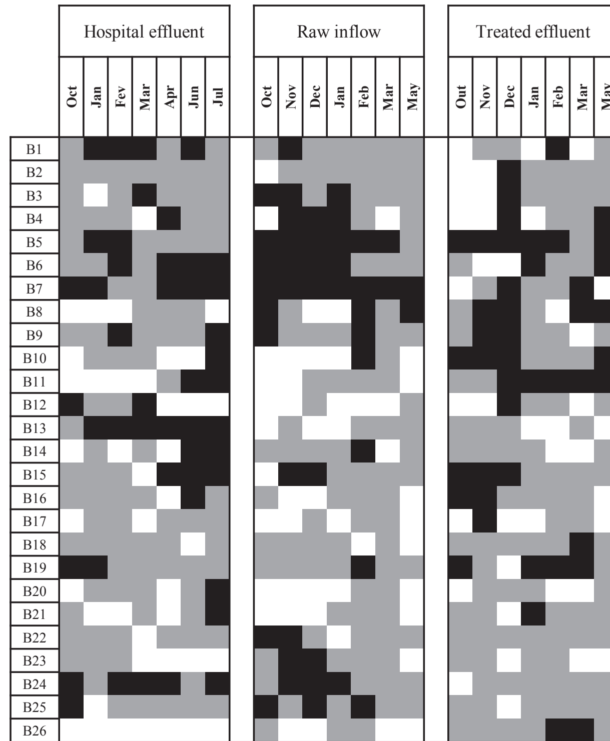

Figure S2. Detrended Correspondence Analysis of the 16S rRNA gene-DGGE profiles of hospital effluent (n = 7, △) and raw inflow (n = 7,●) and treated effluent (n = 7,○) of the respective wastewater treatment plant. The score of variation of the DGGE bands was of 0.615, 19.4% in axis 1 and 10.7% in axis 2. Samples numbering is as described in the legend of Fig. 1.

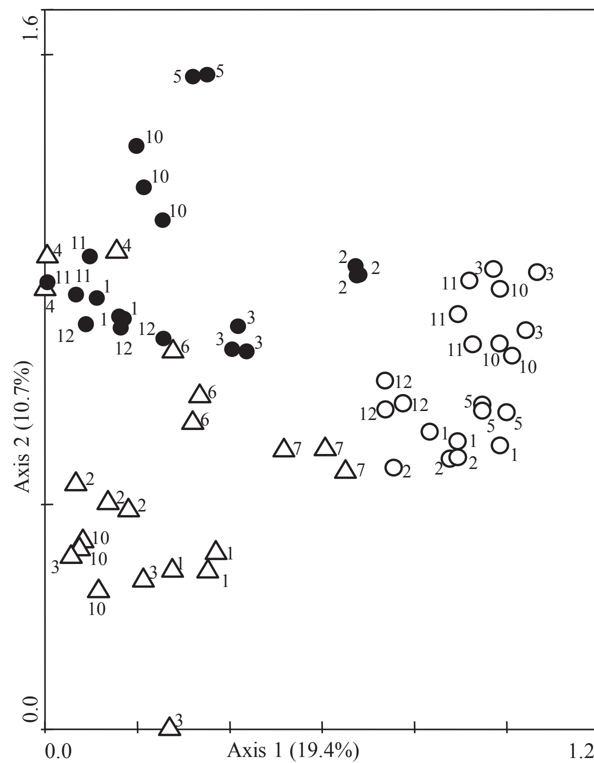

Figure S3. DGGE band occurrence and intensity in the hospital effluent and raw inflow and treated effluent of the respective wastewater treatment plant. Band classes (B1–B26) were classified according to their intensity, in each month of sampling, as: white = absent, grey = weak (with less than 1/2 of the maximum intensity detected) and black = strong (with more than 1/2 of the maximum intensity detected).

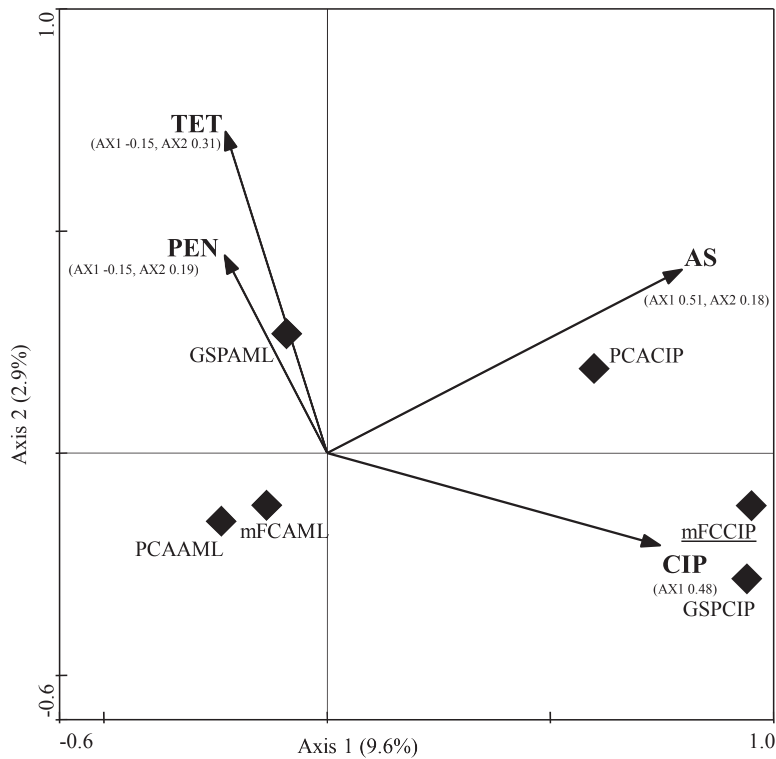

Figure S4A. Correspondence Canonical Analysis (CCA) of the variation of the percentage of antibiotic resistant cultivable bacteria (the score of variation of antibiotic resistant populations (◆) was 0.358, 9.6% of which over axis 1 and 2.9% over axis 2), in function of the concentrations of antimicrobial residues tetracycline (TET, $p = 0.002$), ciprofloxacin (CIP, $p = 0.046$), penicillin (PEN, $p = 0.012$) and arsenic (AS, $p = 0.002$). Only the variables significantly ($p < 0.05$) explaining the observed community variation are shown. The species–environmental correlations for axes 1 and 2 were, respectively, 0.643 and 0.431. Bacterial populations with a fraction of variance over axis 1 higher than 0.2/1 are underlined

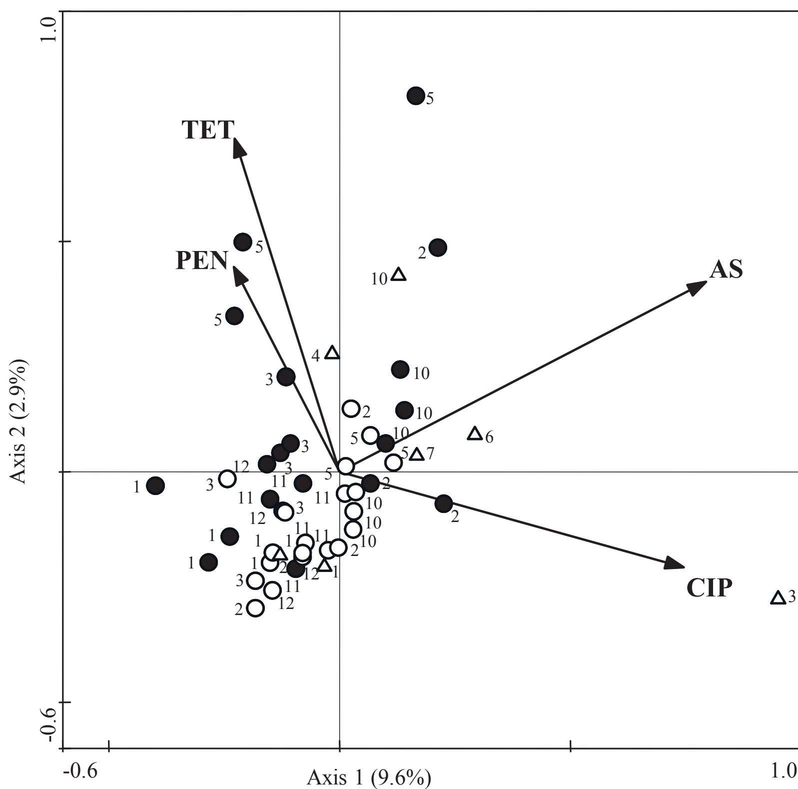

Figure S4B. Correspondence Canonical Analysis (CCA), as described in Figure S4A. Samples of hospital effluent (△), raw inflow (●) and treated effluent (○) of the wastewater treatment plant are numbering according to the legend of Fig. 1.

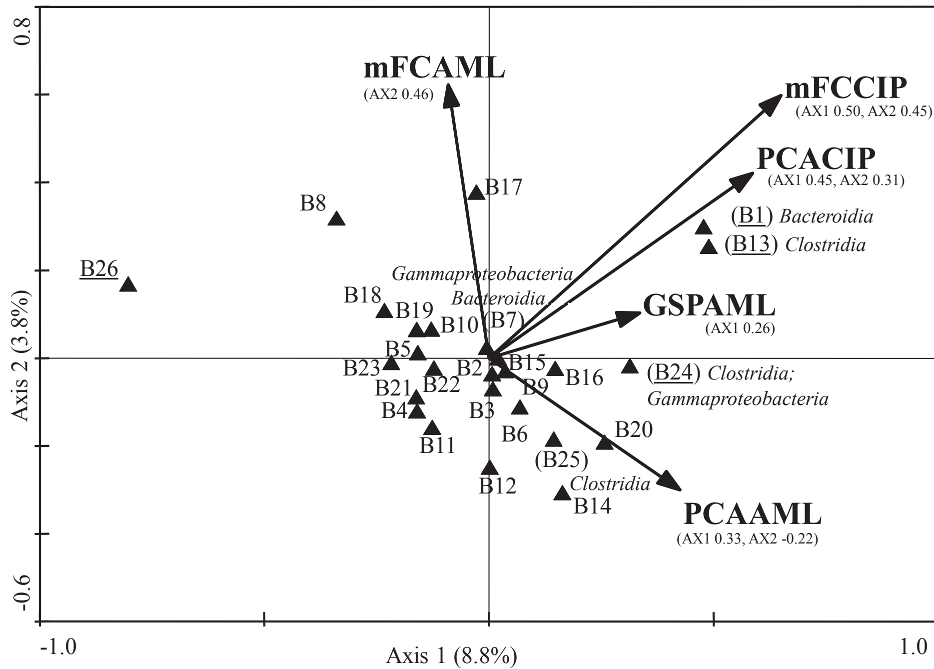

Figure S5A. Correspondence Canonical Analysis (CCA) of the variation of 16S rRNA gene-DGGE patterns of the hospital effluent, wastewater treatment plant raw inflow and treated effluent (the score of variation of the DGGE bands, ▲, was 0.615, 8.8% over axis 1 and 3.8% over axis 2) in function of the percentage of culturable ciprofloxacin resistant heterotrophs (PCACIP, $p = 0.018$) and enterobacteria (mFCCIP, $p = 0.002$), and amoxicillin resistant heterotrophs (PCAAML, $p = 0.038$), aeromonads/pseudomonads (GSPAML, $p = 0.044$) and enterobacteria (mFCAML, $p = 0.002$). Only the variables significantly ($p < 0.05$) explaining the observed community variation are shown. The species–environmental correlations for axes 1 and 2 were, respectively, 0.767 and 0.747. Representation of DGGE bands (▲) distribution; 16S rRNA gene-DGGE bands with a fraction of variance over axis 1 higher than 0.2/1 are underlined.

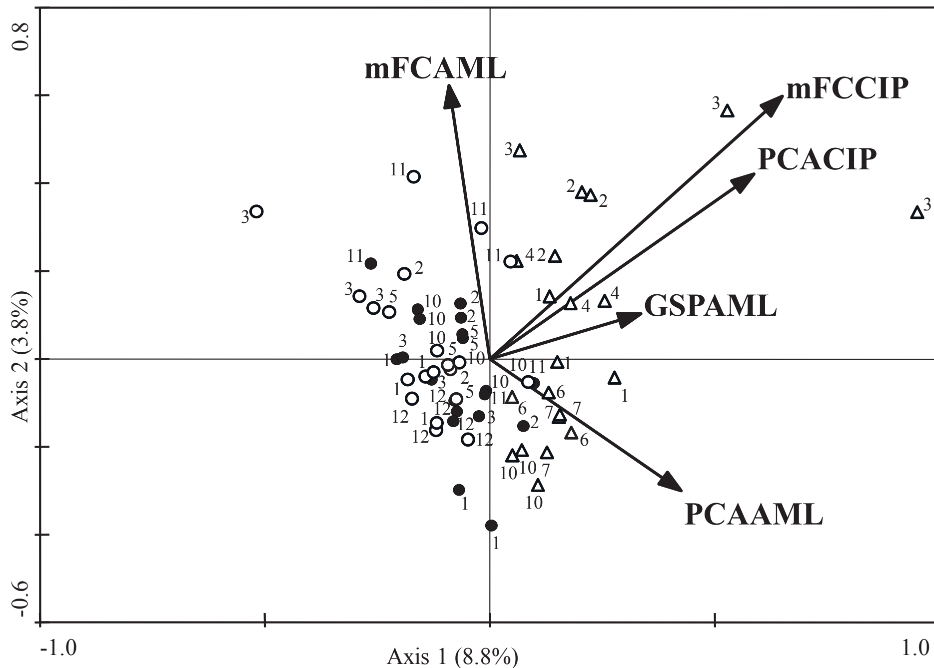

Figure S5B. Correspondence Canonical Analysis (CCA), as described in Figure S5A. Samples of hospital effluent (△), raw inflow (●) and treated effluent (○) of the wastewater treatment plant are numbering according to the legend of Fig. 1.

Chapter 2

Genetic characterization of fluoroquinolone resistant *Escherichia coli* from urban streams and municipal and hospital effluents

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Research Article

RESEARCH ARTICLE

Genetic characterization of fluoroquinolone resistant *Escherichia coli* from urban streams and municipal and hospital effluents

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One sentence summary: Fluoroquinolone resistant lineages of *Escherichia coli* are able to persist in aquatic environments and to spread their resistance determinants through both vertical inheritance and horizontal gene transfer.
Editor: Kornelia Smalla

ABSTRACT

Escherichia coli with reduced susceptibility to ciprofloxacin, isolated from urban streams, wastewater treatment plants and hospital effluent between 2004 and 2012, were compared based on multilocus sequence typing (MLST), quinolone and beta-lactam resistance determinants and plasmid replicon type. Isolates from the different types of water and isolation dates clustered together, suggesting the persistence and capacity to propagate across distinct aquatic environments. The most prevalent MLST groups were ST10 complex and ST131. Almost all isolates (98%) carried mutations in the chromosomal genes *gyrA* and/or *parC*, and 10% possessed the genes *qepA*, *aac(6′)-Ib-cr* and/or *qnrS1*. Over 80% of the isolates were resistant to three or more classes of antibiotics (MDR $\geq$ 3). The most prevalent beta-lactamase encoding gene was bla_{TEM}, followed by $bla_{CTX\text{-}M\text{-}15}$, co-existing with plasmid mediated quinolone resistance. The plasmid replicon types of the group IncF were the most prevalent and distributed by different MLST groups. The genes *aac(6′)-Ib-cr* and/or *qnrS1* could be transferred by conjugation in combination with the genes bla_{TEM},$bla_{SHV\text{-}12}$ or $bla_{OXA\text{-}1}$ and the plasmid replicon types I1-Iγ, K, HI2 and/or B/O. The potential of multidrug resistant *E. coli* with reduced susceptibility to ciprofloxacin, harboring mobile genetic elements and with ability to conjugate and transfer resistance genes, to spread and persist across different aquatic environments was demonstrated.

Keywords: water; quinolone resistance; PMQR; plasmids replicon type; *E. coli* MLST

INTRODUCTION

The increase in the prevalence of antibiotic resistance has come into focus in the latter decades, frequently attributed to the continuous use of antibiotics and other antimicrobials (Martinez 2009; Tello, Austin and Telfer 2012). The occurrence of new types of resistance and new combinations of resistance

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genes is, in general, reported initially in clinical and/or veterinary settings (Acar 1997; Robicsek *et al.* 2006a; Kumarasamy *et al.* 2010) but detection in other environments, in particular in wastewater, is usually reported shortly after (Szczepanowski *et al.* 2009). Wastewater discharges are major sources of antibiotic resistant bacteria and resistance determinants, part of which will spread in the environment (Baquero, Martínez and Cantón 2008; Martinez 2009; Rizzo *et al.* 2013; Vaz-Moreira, Nunes and Manaia 2014). Although the fate of antibiotic resistance genes in the environment is not completely understood (Martínez and Baquero 2014), it has been demonstrated that some persist even in the absence of selective pressures (Andersson and Hughes 2012). Quinolones, antimicrobial therapeutic agents introduced in 1962, are a group of broad spectrum synthetic antibiotics used for a variety of infections. These drugs were expected to bypass the problem of resistant bacteria, since no resistance mechanism specific for this new class of antibiotics was supposed to exist in nature (Robicsek, Jacoby and Hooper 2006a). However, following quinolone introduction into the clinical practice, resistance to this class of antibiotics became commonly reported in diverse environments (Martínez-Martínez, Pascual and Jacoby 1998; Novo and Manaia 2010; Mokracka *et al.* 2011; Vredenburg *et al.* 2013). In *Enterobacteriaceae,* quinolone resistance emerged independently several times (Strahilevitz *et al.* 2009), and is associated with chromosome mutations and/or with plasmid-borne genes. The most frequent mechanism of resistance to quinolones in *Escherichia coli* includes mutations in the quinolone resistance-determining region of the genes *gyrA* and *parC* (Heisig 1996; Webber *et al.* 2013). Plasmid-associated quinolone resistance genes have also been described (Park *et al.* 2006; Cattoir *et al.* 2007; Périchon, Courvalin and Galimand 2007; Cavaco *et al.* 2009; Wang *et al.* 2009). Although these genes confer only low-level resistance, it has been argued that their presence can favor the occurrence of chromosomal mutations, which will increase the minimal inhibitory concentration tolerated and, therefore, the resistance level (Robicsek *et al.* 2006b).

Conjugative plasmids are important elements for horizontal gene transfer, contributing to spread antibiotic resistance genes (Carattoli 2009; Parsley *et al.* 2010; Villa *et al.* 2010). For this reason, plasmids of ubiquitous and human-associated bacteria are regarded as interesting tools to track antibiotic resistance acquisition (Carattoli 2009). On the other hand, *E. coli*, a universal indicator of fecal contamination due to its anthropogenic origin, is one of the most interesting bacterial groups to infer about the processes and paths of resistance dissemination. Members of this species harbor a myriad of plasmids, known to be associated with the capacity to acquire different families of resistance genes (Mammeri *et al.* 2005; Carattoli 2009; Shibl *et al.* 2012). Simultaneously, the emergence of virulent and multidrug resistant (MDR) *E. coli* strains (French 2010; Woodford, Turton and Livermore 2011) highlights the importance of monitoring antibiotic resistance determinants in environmental populations of these bacteria. *E. coli* sequence type (ST) 131 is of this a good example, since members of this group frequently combine virulence and multidrug resistance (Nicolas-Chanoine *et al.* 2008; Johnson *et al.* 2010; Rogers, Sidjabat and Paterson 2011).

In previous studies, we demonstrated that quinolone resistance was highly prevalent in Gram-negative bacteria (*E. coli* and *Aeromonas*) isolated from wastewater and that wastewater treatment could select for ciprofloxacin resistant *E. coli* (Ferreira da Silva *et al.* 2007; Figueira, Serra and Manaia 2011a; Figueira *et al.* 2011b). These results motivated a further research on the genetic characterization of fluoroquinolone resistant *E. coli* from waters with fecal contamination, such as urban streams, hospital effluents (HEs) and municipal wastewater, isolated over a period of nine years. In particular, we aimed to identify the genetic determinants of quinolone resistance in these isolates, the weight of vertical and horizontal transmission of this type of resistance and to assess if quinolone and beta-lactam resistance determinants in *E. coli* are transmitted simultaneously in the environment. Using multilocus sequence typing (MLST), plasmid replicon typing, antibiotic resistance phenotyping, quinolone and beta-lactam resistance genes detection and conjugation experiments, it was intended to assess: (a) the major quinolone resistance genes in these isolates and the associated beta-lactamase genes and plasmid replicons; (b) if specific MLST groups were associated with a given source, resistance profile or plasmid carriage; (c) if acquired quinolone resistance genes were harbored by conjugative plasmids.

METHODS

Bacterial isolates

A collection of 80 ciprofloxacin resistant *E. coli* isolates from raw wastewater (RWW, $n = 28$) and treated wastewater (TWW, $n = 33$) of four urban wastewater treatment plants, from urban streams and ponds (US, $n = 9$), herein designated as streams, and from untreated HE ($n = 10$) was analyzed (Fig. S1, Supporting Information). The collection comprised isolates recovered between 2004 and 2012, on antibiotic-free culture media ($n = 29$) or on the same media supplemented with 4 mg L^{-1} ciprofloxacin ($n = 45$) or other antibiotics (16 mg L^{-1} tetracycline, $n = 1$; 32 mg L^{-1} amoxicillin, $n = 1$; 350 mg L^{-1} sulfamethoxazole, $n = 4$), as described in previous studies (Ferreira da Silva *et al.* 2007; Figueira, Serra and Manaia 2011a; Novo *et al.* 2013; Varela *et al.* 2014).

MLST analysis

The isolates were genotyped by MLST, based on the housekeeping genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* as proposed by Wirth *et al.* (2006). Allelic profiles and STs were determined based on *E. coli* MLST database queries (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). Phylogenetic analysis was carried out using the concatenated sequences of the seven genes. Sequences were aligned using ClustalW and dendrograms were constructed using MEGA version 6 (Tamura *et al.* 2013). The evolutionary distances, estimated based on the model of Jukes and Cantor (1969). The dendrogram was constructed based on neighbor-joining method and its stability was confirmed with the maximum likelihood method, using MEGA version 6 (Tamura, Nei and Kumar 2004, 2013).

Detection of plasmid replicons

The presence of plasmid replicons was determined as described by Carattoli *et al.* (2005), for FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P,W, T, A/C, K, B/O, X and Y, and by Villa *et al.* (2010) for the FII type. Positive controls were included in each PCR assay and the authenticity of the test amplicons was confirmed based on nucleotide sequence analysis and query of public databases (http://www.ncbi.nlm.nih.gov/blast/). Isolates yielding replicons of the groups FIA, FIB and/or FII and plasmid mediated quinolone resistance or beta-lactamase genes (described below) were further characterized by plasmid MLST (http://pubmlst.org/plasmid/) (Villa *et al.* 2010).

Antibiotic resistance phenotypes

Antibiotic resistance phenotypes were determined based on the disk diffusion method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2012). Disks of amoxicillin (AML, 25 μg), ticarcillin (TIC, 75 μg), cephalothin (CP, 30 μg), ceftazidime (CAZ, 30 μg), meropenem (MEM, 10 μg), colistin (CT, 50 μg), sulfamethoxazole (SUL, 25 μg), sulfamethoxazole/trimethoprim (SXT, 25 μg), ciprofloxacin (CIP, 5 μg), tetracycline (TET, 30 μg), gentamycin (GEN, 10 μg) or streptomycin (STR, 10 μg) (Oxoid) were dispensed on Mueller-Hinton agar cultures, which incubated at 37°C for 24 h. The MIC (minimum inhibitory concentration) of ciprofloxacin was determined for selected isolates using strips embedded with an antibiotic gradient (MIC Evaluator, Oxoid). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* DSM 1117 were included as quality control strains in all assays. Were considered MDR isolates exhibiting resistance to three or more different classes of antibiotics (penicillins, cephalosporins, carbapenems, aminoglycosides, quinolones, sulfonamides, tetracyclines, polymyxins) (Magiorakos *et al.* 2012).

Genetic determinants associated with ciprofloxacin or beta-lactam resistance

Chromosomal mutations in the genes *gyrA* and *parC* were analyzed in the nucleotide sequences of PCR amplicons generated with the primers gyrA6/gyrA631R and HJL3/HJL4, respectively, as described previously (Weigel *et al.* 1998; McDonald *et al.* 2001).

The detection of the genes *qnrA, qnrB, qnrC, qnrD, qnrS, qepA, qnrVC* and *aac(6′)-Ib-cr*, encoding plasmid-mediated quinolone resistance, was performed as described by Figueira *et al.* (2011a,b) and Tacão *et al.* (2014). Isolates were also screened for the presence of the genes *oqx*AB, using the protocol described by Liu *et al.* (2011), with an annealing temperature of 55°C. The following references were included in each PCR reaction and nucleotide sequence analysis as positive and quality control: *E. coli* L0 (*qnrA1*+), *Klebsiella pneumoniae* B1 (*qnrB1*+), *Enterobacter cloacae* S1 (*qnrS1*+) (Cattoir *et al.* 2007), *E. coli* TOP10+ paT851 (*qepA*+) (Périchon, Courvalin and Galimand 2007), *E. coli* DH10B transformant pHS11 (*qnrC*+) (Wang *et al.* 2009), *E. coli* DH10B transformant p2007057 (*qnrD*+) (Cavaco *et al.* 2009), *oqx*A+ and *oqx*B+ isolates from the laboratory's culture collection. The gene *aac(6′)-Ib* was further inspected through nucleotide sequence analysis for detection of the mutations that characterize the *aac(6′)-Ib-cr* variant (Robicsek *et al.* 2006b).

Beta-lactamase encoding genes commonly found in *E. coli* were also surveyed by PCR and nucleotide sequence determination. Primer sets and PCR conditions for bla_{OXA} (Ouellette, Bissonnette and Roy 1987; Henriques *et al.* 2006), bla_{TEM} and bla_{SHV} (DiPersio *et al.* 2005) and $bla_{CTX\text{-}M}$ and $bla_{OXY\text{-}2}$ (Weill *et al.* 2004; Monstein, Tärnberg and Nilsson 2009) were the same as reported in previous publications. The presence of beta-lactamase encoding genes from the *ampC* family (bla_{CMY} and bla_{LAT}) was also examined by PCR (Dierikx *et al.* 2010). Briefly, for these genes the following conditions were used for reactions of 25 μL: 1 μL DNA, 1 U of *Taq* DNA polymerase (ThermoScientific), 1 × KCl PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP's mix (ThermoScientific) and 20 pmol of each primer (5′-ATGATGAAAAAATCGTTATGCTGC-3′ and 5′-GCTTTTCAAGAATGCGCCAGG-3′), and a thermal cycling program consisting of 5 min at 94°C, 30 cycles of 1 min at 92°C, 1 min at 58°C and 1 min at 72°C and a final extension step of 10 min at 72°C (Biometra). Positive controls from the laboratory's culture collection were included in the PCR assays, and PCR product authenticity was confirmed based on nucleotide sequence analysis and database query, using BLAST (http://www.ncbi.nlm.nih.gov/blast/) and the database at http://www.lahey.org /Studies/.

Conjugation assays

Isolates carrying plasmid-encoded quinolone resistance (PMQR) were further characterized for their ability to transfer resistance by conjugation, using the sodium azide resistant *E. coli* J53 as the recipient strain. Culture of donors and recipient strains grown in Luria-Bertani (LB) broth for 4 h were mixed in equal volumes, centrifuged at 10 000 rpm for 5 min, suspended in the same medium and incubated overnight at 28°C. Putative transconjugants were selected on LB agar plates supplemented with sodium azide (100 mg L^{-1}) and amoxicillin (32 mg L^{-1}) or ciprofloxacin (0.06 mg L^{-1}). Transconjugants were confirmed through random amplified polymorphic DNA genotyping and screened for the antibiotic susceptibility phenotypes, the presence of plasmid replicon types and resistance determinants detected in the donor strains, as described above.

Statistical analyses

The prevalence of antibiotic resistance and plasmid replicon type was compared among MLST groups and isolates recovered on ciprofloxacin-free/supplemented media, using Monte Carlo simulations of Fisher's exact test based on sampling 10 000 tables at a significance level (P) of <0.05. All statistical analyses were performed using SPSS 19.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Genotypic diversity

MLST analysis was used as a method to assess possible paths of transmission of fluoroquinolone resistant *E. coli* in waters with fecal contamination. From a collection of 776 *E. coli* isolates (Figueira, Serra and Manaia 2011a, Varela *et al.* 2014) recovered over a period of nine years from municipal wastewater, HE and urban streams 80 isolates with reduced susceptibility to ciprofloxacin were selected for further comparison. Most of the isolates yielded sequences matching 32 STs of the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). The most commonly found types were ST10 complex (13 isolates) and ST131 (seven isolates). The combination of the ST determined for the seven alleles of five isolates did not match any entry in the database. These sequences were submitted as new STs, with the numbers (ST4460, ST4656, ST4659, ST4703 and ST4712).

Concatenated sequences corresponding to 3386 nucleotide positions were compared, leading to the establishment of five groups (A–E), formed at sequence identity values above 99% (more than 3352 identities). Three isolates that shared among them a similarity value of 98.6% formed an additional group, which given the small dimension was herein considered of unclustered isolates. Groups A, B and D formed a major cluster (Fig. 1). Group A included 25 isolates, mostly from TWW but also from HE, RWW and urban streams. In group A, isolates were predominantly related with complex ST10, including ST10 and ST44. Group B was composed of eight isolates from urban wastewater treatment plants (TWW and RWW), related with the

complexes ST469, ST46 and ST398. Group C included 10 isolates originated from all types of water sampled and mostly related with the ST131. Group D comprised 26 isolates also from all types of water and distributed by 18 ST. This group was the most genetically diverse and included bacteria related with the complexes ST155, ST156, ST205, ST40 and ST101. Group E was composed of eight isolates from urban wastewater and urban streams, related with the complex ST354. The three unclustered isolates were from RWW and TWW, and were related with the complexes ST31 and ST69. The frequency of isolates from ciprofloxacin-supplemented or antibiotic-free culture medium in each MLST group was non-significantly different ($P > 0.05$), suggesting that those isolation conditions did not influence the genetic diversity of the *E. coli* isolates recovered.

Plasmid replicon typing

Since plasmids are considered major drivers in processes of horizontal gene transfer, we were interested in characterizing the pattern of plasmid replicons in function of the phylogenetic diversity. It was possible to identify 12 plasmid replicon types out of the 18 that were screened for, suggesting a wide potential for propagation through horizontal gene transfer. The observed replicon types were randomly distributed by the different phylogenetic lineages and types of water (Fig. 1). The most prevalent replicon types were FII (53/80; 66.3%) and FIB (49/80; 61.3%), whereas all the others presented prevalence values below 26.5% (Table 1). The isolation conditions seemed to be related to the presence of the replicon type N ($n = 9$), which was only detected

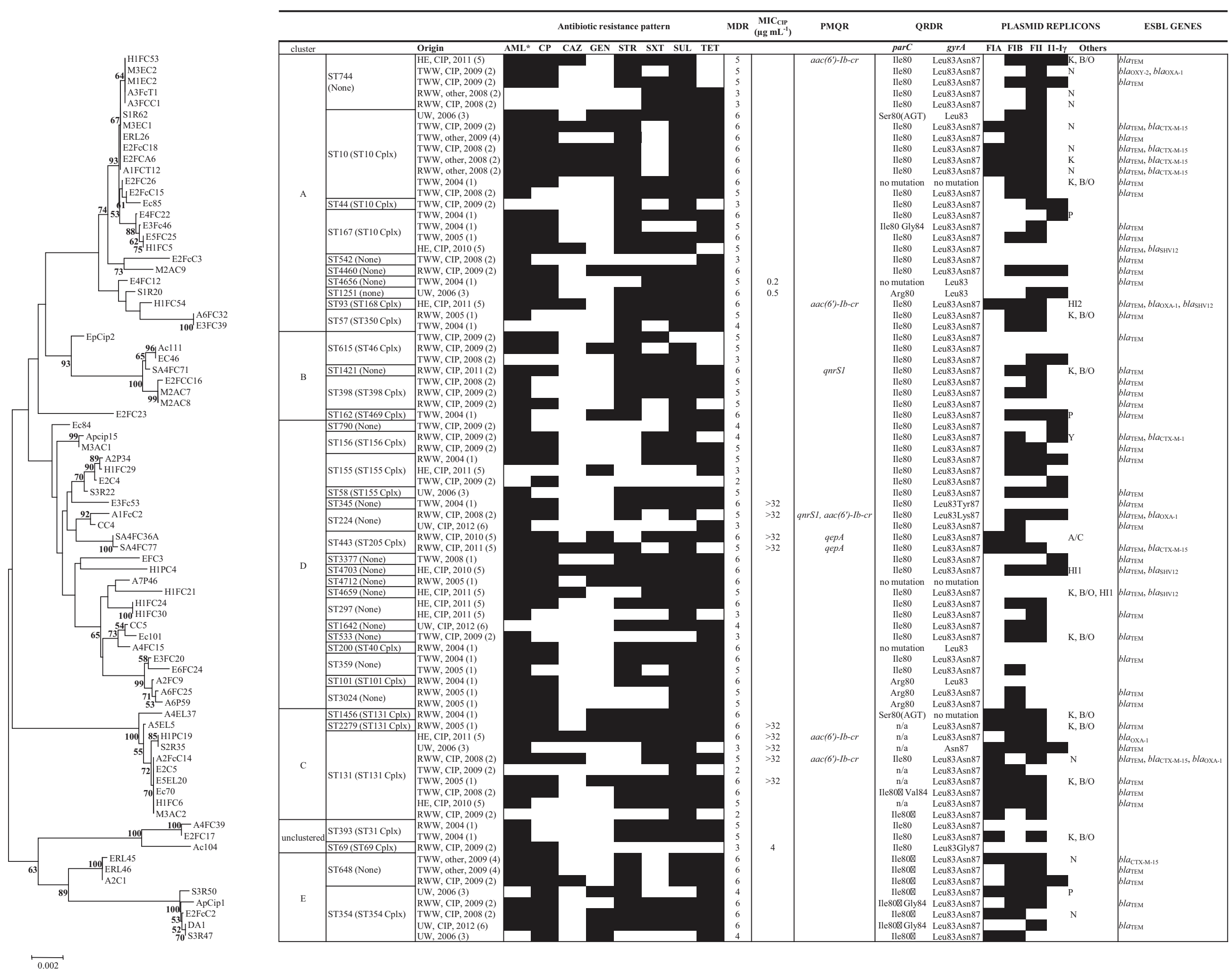

Figure 1. Neighbor-joining dendrogram based on the concatenated nucleotide sequences of the housekeeping genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* (number of positions = 3386) of *E. coli* strains with reduced susceptibility to quinolones. Bootstrap values greater than 50% based on 1000 replications are given at the internal nodes. Black dots indicate branches recovered also based on the maximum likelihood method. Origin: HE, HE, RWW and TWW, urban wastewater treatment plant raw inflow and TWW, respectively, US, urban streams; CIP, recovered in media supplemented with 4 mg L^{-1} ciprofloxacin; other, recovered in media supplemented with other antibiotics (32 mg L^{-1} amoxicillin, 16 mg L^{-1} tetracycline or 350 mg L^{-1} sulfamethoxazole); AML, amoxicillin; TIC, ticarcillin; CP, cephalothin, CAZ, ceftazidime; GEN, gentamycin; STR, streptomycin; SXT, sulfamethoxazole/trimethoprim; SUL, sulfamethoxazole; TET, tetracycline. MDR, number of classes of antibiotics to which resistance was detected. *Resistance to AML and TIC was similar for all isolates. All isolates were susceptible to meropenem and colistin. n/a, no sequence available. Ser80(AGT), silent mutation; a, AGC → ATT mutation. (1) Ferreira da Silva *et al.* (2007); (2) Novo and Manaia (2010); (3) Figueira *et al.* (2011a), (4) Varela *et al.* (2014); (5) this study.

Table 1. Prevalence of antibiotic resistance and/or plasmid-carriage in isolates of each MLST group.

MLST groups	Plasmid replicons (%)								Antibiotic resistance (%)									
	FIA ($n = 21$)	FIB ($n = 49$)	FII ($n = 53$)	N ($n = 9$)	I1-Iγ ($n = 18$)	K ($n = 11$)	B/O ($n = 10$)	Other[b]	AML ($n = 68$)	TIC ($n = 68$)	CP ($n = 55$)	CAZ ($n = 13$)	GEN ($n = 21$)	STR ($n = 57$)	SXT ($n = 48$)	SUL ($n = 70$)	TET ($n = 62$)	MDR4 ($n = 66$)
A ($n = 25$)	20.0	60.0	84.0	24.0	24.0	16.0	12.0	P, HI2	84.0	84.0	72.0	28.0	24.0	84.0	60.0	92.0	80.0	84.0
B ($n = 8$)	0.0	37.5	62.5	0.0	25.0	12.5	12.5	P	87.5	87.5	50.0	0.0	25.0	100.0	62.5	87.5	50.0	87.5
C ($n = 10$)	88.9[a]	100.0	100.0	11.1	11.1	33.3	33.3	–	80.0	80.0	60.0	20.0	40.0	60.0	80.0	100.0	70.0	70.0
D ($n = 26$)	7.7	61.5	42.3[a]	0.0	30.8	7.7	7.7	Y, HI1, A/C	88.5	88.5	69.2	11.5	19.2	50.0[a]	57.7	84.6	80.8	76.9
E ($n = 8$)	50.0	87.5	75.0	25.0	12.5	0.0	0.0	P	75.0	75.0	100.0	12.5	50.0	87.5	37.5	75.0	100.0	100.0
other ($n = 3$)	33.3	0.0	33.3	0.0	0.0	33.3	33.3	–	100.0	100.0	33.3	0.0	0.0	66.7	66.7	66.7	66.7	66.7
Total ($n = 80$)	26.3	61.3	66.3	11.3	22.5	13.8	12.5		85.0	85.0	68.8	16.3	26.3	71.3	60.0	87.5	77.5	81.3

AML, amoxicillin; TIC, ticarcillin; CP, cephalotin, CAZ, ceftazidime; GEN, gentamycin; STR, streptomycin; SXT, sulfamethoxazole/trimethoprim; SUL, sulfamethoxazole; TET, tetracycline. All isolates were susceptible to meropenem and colistin; MDR4, resistant to 4 or more classes of antibiotics; a, significantly different ($p < 0.05$) from the other groups; b, less than 3% of the plasmid replicons detected.

in isolates recovered on culture-medium with ciprofloxacin. By contrast, the presence of ciprofloxacin in the isolation culture medium was not associated with any significant differences ($P > 0.05$) in the prevalence of plasmid replicon types FIA, FIB, FII, I1-Iγ, K and B/O. In general, the most prevalent replicon types were randomly distributed by the different MLST groups. The only exceptions were the replicon type FIA, which was significantly ($P < 0.05$) more prevalent in group C (related with ST131) and the replicon type FII, significantly less prevalent ($P < 0.05$) in group D (related with ST155, ST156, ST297, ST443 and ST3024) (Table 1). Nucleotide sequences representative of each type of plasmid replicon found were deposited in the GenBank (Accession numbers LN714727 – 38).

Antibiotic resistance phenotypes

Resistance phenotypes were determined for antibiotics representative of eight classes of antibiotics. Most isolates were resistant to sulfamethoxazole (70/80), amoxicillin and ticarcillin (68/80), and tetracycline (62/80). The prevalence of resistance to ceftazidime, comparatively lower than resistance to other antibiotics ($n = 13/80$), was observed mainly in HE isolates ($P < 0.05$, 6/10) and absent in stream isolates. Although resistance phenotypes to meropenem and colistin were not observed, resistance to four or more classes of antibiotics (MDR4, including fluoroquinolones) was highly prevalent (82.1% in RWW, 81.8% in TWW, 80.0% in HE). In order to infer about the possible co-selection of resistance to fluoroquinolone and other antibiotics, the prevalence of resistance among isolates recovered on ciprofloxacin-supplemented ($n = 45$) and antibiotic-free culture medium ($n = 29$) was compared. The co-selection hypothesis was confirmed for ceftazidime resistance, which was found to be significantly ($P < 0.05$) more prevalent among isolates recovered on ciprofloxacin supplemented than from antibiotic-free culture medium (11/45 versus 2/29). By contrast, the isolation of tetracycline resistant and multidrug resistant bacteria (MDR4) was significantly ($P < 0.05$) less frequent on ciprofloxacin-supplemented culture medium (17/45 and 13/32, respectively). When antibiotic resistance prevalence was compared among the five MLST groups (A–E), the only significant ($P < 0.05$) difference was the lower percentage of streptomycin resistance in group D (Table 1). Curiously, group D had also the lowest prevalence of replicon type FII.

Chromosomal determinants of quinolone resistance

Mutations in the chromosomal genes *gyrA* and/or *parC* could explain the reduced ciprofloxacin susceptibility observed in 77 of the 80 isolates. A total of 76 isolates showed an amino acid exchange in the position 83 of the DNA gyrase, with the substitution of serine by leucine (TCG→TTG), either alone ($n = 5$) or combined with an additional mutation in the position 87. This second mutation consisted in the substitution of the aspartate by asparagine (GAC→ AAC, $n = 70$), glycine (GGC, $n = 1$) or tyrosine (TAC, $n = 1$) (Fig. 1). For a single isolate, this was the only mutation detected in *gyrA*. A total of 70 isolates also carried mutations in the gene *parC* (Fig. 1), the most common occurring in position 80 and consisting of the substitution of the serine by isoleucine (AGC →ATC, $n = 54$; AGC →ATT, $n = 10$), or less frequently by an arginine (AGC →AGA). In addition, two isolates presented a silent mutation in this position (AGC→AGT). Four isolates presented an additional mutation at position 84, in which glutamate was substituted by glycine (GGA, $n = 3$) or valine (GTA, $n = 1$). All isolates of group E presented the codon ATT for isoleucine instead of the more common ATC for in

position 80. In two isolates, from RWW and TWW, neither *gyrA* nor *parC* yielded mutations associated with quinolone resistance. For six isolates, all clustered in group C and mostly related with the ST131, it was not possible to obtain pure sequences of the gene *parC* in spite of the multiple attempts made, suggesting a mutation in the area targeted by the primers. Curiously, all these isolates displayed MIC_{CIP} values higher than 32 μg mL^{-1} independently of the coupled *gyrA* mutations or presence of PMQR genes. It is worth of notice that isolates without mutations or carrying the gyrase mutations leucine (TTG) at position 83, tyrosine (TAC) or asparagine (AAC) at position 87, were recovered only from antibiotic-free culture medium, which indicates that these mutation profiles may be related to the respective MIC_{CIP} values of <4 μg mL^{-1} for the substitution Leu83Gly87 and >32 μg mL^{-1} for the substitution Leu83Tyr87.

Plasmid-encoded quinolone resistance

PMQR was detected in 10% (8/80) of the isolates from RWW and HE, but not in isolates from treated or urban streams. These genes were detected in isolates presenting quinolone resistance-conferring *gyrA* and/or *parC* mutations, and were only found in isolates from ciprofloxacin-supplemented culture medium. Most (7/8) isolates carrying PMQR displayed an elevated MIC_{CIP} of >32 μg mL^{-1}, although an identical MIC value could be observed in isolates that did not harbor any of the PMQR investigated (Fig. 1; Table 2). All eight isolates with PMQR were resistant to five or more classes of antibiotics (MDR5, $n = 4$; MDR6, $n = 4$) (Table 2). The gene a*ac(6′)-Ib-cr* was the most common among the PMQR determinants detected, and exclusively found in isolates from HE and RWW, related with ST93, ST744 and ST131. Two variants of this gene, with the transition TGG→CGG or the transversion TGG→AGG in the codon 102 were observed irrespective of the origin of the isolates. A single RWW isolate yielded the gene *aac(6′)-Ib* without any of the three mutations conferring resistance to quinolones. The genes *qepA* and *qnrS1* were detected only in RWW isolates, with *qnrS1* co-occurring with *aac(6′)-Ib-cr.* The gene *qepA* was detected in two isolates of the complex ST205 that in spite of high genotypic resemblance presented distinct resistance profiles and genetic determinants, evidencing processes of horizontal gene transfer. Both *qepA* gene sequences were identical, displaying neutral substitutions of phenylalanine (TTC) in position 95 for leucine (TTA), differing from *qepA1* (FJ744121.1) and *qepA2* (EU847537.1), and of valine (GTC) in position 134 for isoleucine (ATC), identical to *qepA1*. Novel *qepA* nucleotide sequences were deposited in the GenBank (Accession numbers LK934677-8). The genes *qnrA*, *qnrB*, *qnrC, qnrD, qnrVC* and *oqx*AB were not detected in any of the isolates.

All strains with PMQR presented plasmid replicons from the IncF group, as well as other replicon types such as I1-Iγ, B/O, K, HI2, N and A/C. According to the replicon MLST analysis, two isolates recovered from different sources in separate years (HE 2011, and RWW 2008) shared the allele combination FIA-; FIB1; FII24. Despite this similarity of plasmid replicon types, these isolates harbored different variants of the gene *aac(6′)-Ib-cr,* and one (from RWW) carried also the gene *qnrS1,* suggesting possible recombination events (Table 2). Moreover, hinting at possible horizontal gene transfer, both isolates with identical plasmid replicons were genetically distinct, belonging to different clusters (A and D) and different lineages (ST744 and ST224, respectively).

Characterization of beta-lactamase-encoding genes

Since beta-lactam resistance was observed in 85% of the isolates and plasmid encoded beta-lactamases are often reported in quinolone resistant *E. coli*, genes related with beta-lactam resistance were also searched in these isolates. Genes encoding beta-lactamases were detected in 52 isolates. All isolates with PMQR presented at least one of the surveyed beta-lactamase encoding genes. The gene bla_{TEM} was the most common, being present in isolates from all types of water and isolation conditions. Isolates carrying the gene $bla_{CTX\text{-}M}$ were all from urban wastewater and yielded MDR4 to MDR6 profiles. The variant $bla_{CTX\text{-}M\text{-}15}$ was predominant (7/8) and in two isolates of the ST131 and ST205 complex, and co-existed with the genes *aac(6′)-Ib-cr,* $bla_{OXA\text{-}1}$, and *qepA*, respectively. The gene $bla_{OXA\text{-}1}$ was observed in five isolates, distributed by all MLST clusters and types of wastewater, co-existing with the genes $bla_{OXY\text{-}2}$, $bla_{CTX\text{-}M\text{-}15}$, bla_{SHV} or with PMQR genes (*qnrS1, aac(6′)-Ib-cr*) (Fig. 1). The gene $bla_{SHV\text{-}12}$ was only detected in HE ($n = 4$), in isolates distributed by different MLST groups, and, in one isolate, co-existed with the gene *aac(6′)-Ib-cr*. The genes $bla_{CTX\text{-}M\text{-}15}$, $bla_{SHV\text{-}12}$ and $bla_{OXA\text{-}1}$ were associated with the ceftazidime resistance phenotypes observed. Plasmid encoded beta-lactamase genes of the *ampC* family was not detected.

Conjugative transfer of PMQR or beta-lactamase genes

The eight isolates harboring PMQR were tested for their conjugation capacity with the strain *E. coli* J53 as recipient and selection of transconjugants on culture medium with 32 mg L^{-1} amoxicillin or 0.06 mg L^{-1} ciprofloxacin (Table 3). Selection on amoxicillin-supplemented culture medium allowed the recovery of five transconjugants and on ciprofloxacin of four. Of the six strains originating transconjugants, three were either recovered on amoxicillin or on ciprofloxacin-supplemented culture medium, and presented different characteristics for both selection conditions (Table 3). All amoxicillin transconjugants showed acquired phenotypes of resistance to amoxicillin, ticarcillin and sulfamethoxazole. Resistance acquired by transconjugants obtained on culture medium with ciprofloxacin varied between no acquired resistance and resistance to sulfamethoxazole, to amoxicillin/ticarcillin and to tetracycline. Plasmid replicon types acquired by conjugation comprised FIA, FII, I1-Iγ, K, B/O, HI2 and N, and among the beta-lactamase genes, bla_{TEM}, bla_{SHV} and bla_{OXA} were transferred (Table 3). Transfer of PMQR was observed for three isolates from HE and RWW, selected on amoxicillin and ciprofloxacin. The gene *qnrS1* was transferred with the gene bla_{TEM} and the replicon types K, B/O and I1-Iγ, and the gene *aac(6′)-Ib-cr* with the genes $bla_{OXA\text{-}1}$, bla_{SHV} and the replicon type HI2. Some genetic elements could only be detected in transconjugants obtained in medium with ciprofloxacin (bla_{OXA}, plasmid replicon B/O) or with amoxicillin (plasmid replicon I1-Iγ).

DISCUSSION

E. coli has been frequently investigated as an indicator of antibiotic resistance in the environment (e.g. Ferreira da Silva et al. 2007; Vredenburg *et al.* 2013; Amos *et al.* 2014). The combination of approaches which investigate the phylogenetic diversity of the bacterial hosts and simultaneously the genetic elements of resistance may bring new insights into several aspects related with antibiotic resistance ecology. This was the basis for the design of this work, aiming at comparing *E. coli* isolates with

Table 2. Genetic characteristics of isolates harboring plasmid mediated quinolone resistance genes.

Origin	Group	ST (ST Cplx)	*parC*	*gyrA*	FAB formula other plasmid replicons	Plasmid resistance genes	*aac(6′)-Ib-cr* mutations	Antibiotic resistance pattern	MDR	MIC_{CIP} ($\mu g\ mL^{-1}$)	Isolate
HE	A	ST744 (None)	Ile80	Leu83Asn87	F24:A-:B1 I1-Iγ, K, B/O	*aac(6′)-Ib-cr* bla_{TEM}	102:CGG,117:TTA,179:TAT	AML, TIC/CP, CAZ/ STR/SXT, SUL	5	8	H1FC53
HE	A	ST93 (ST168 Cplx)	Ile80	Leu83Asn87	F18:A1:B1 HI2	*aac(6′)-Ib-cr* bla_{TEM} bla_{OXA-1} bla_{SHV-12}	102:AGG,117:TTA,179:TAT	AML, TIC/CP, CAZ/ STR/SXT, SUL/TET	6	>32	H1FC54
HE	C	ST131 (ST131 Cplx)	n/a	Leu83Asn87	F31:A-:B1 P	*aac(6′)-Ib-cr* bla_{OXA-1}	102:CGG,117:TTA,179:TAT	AML, TIC/CP, CAZ/GEN, STR/SXT, SUL/TET	6	>32	H1PC19
RWW	B	ST1421 (None)	Ile80	Leu83Asn87	F18:A-:B8 K, B/O	*qnrS1* bla_{TEM}	–	AML, TIC/CP/STR/SXT, SUL/TET	6	>32	SA4FC71
RWW	C	ST131 (ST131 Cplx)	Ile80	Leu83Asn87	F40:A1:B-N	*aac(6′)-Ib-cr* bla_{TEM} $bla_{CTX-M-15}$ bla_{OXA-1}	102:CGG,117:TTA,179:TAT	AML, TIC/CP, CAZ/SXT, SUL/TET	5	>32	A2FCC14
RWW	D	ST224 (None)	Ile80	Leu83Asn87	F24:A-:B1 I1-Iγ	*qnrS1*, *aac(6′)-Ib-cr* bla_{TEM} bla_{OXA-1}	102:AGG,117:TTA,179:TAT	AML, TIC/CP/STR/SUL	5	>32	A1FCC2
RWW	D	ST443 (ST205 Cplx)	Ile80	Leu83Asn87	F-:A1:B26 A/C	*qepA*	–	AML, TIC/CP/GEN/SXT, SUL/TET	6	>32	SA4FC36A
RWW	D	ST443 (ST205 Cplx)	Ile80	Leu83Asn87	F1:A1:B1	*qepA* bla_{TEM} $bla_{CTX-M-15}$	–	AML, TIC/CP, CAZ/GEN, STR/SUL	5	>32	SA4FC77

All isolates were susceptible to meropenem and colistin; MDR, number of classes of antibiotics to which a resistance phenotype was found; n/a, no sequence available. HE – hospital effluent, RWW and TWW – raw inflow and treated wastewater from wastewater treatment plants.

Table 3. Characteristics of transconjugants from isolates harboring plasmid mediated quinolone resistance genes selected on amoxicillin or ciprofloxacin.

Isolate Transconjugant	Selection on	origin	ST/ST cmplx	Antibiotic resistance pattern: AML/TIC	CP	CAZ	GEN	STR	SXT	SUL	TET	MIC_{CIP}* ($\mu g\ mL^{-1}$)	Plasmid replicons	PMQR	Betalactamases
H1FC53		HE	ST744 (None)	R	R	R	S	R	R	R	S	8	FIB, FII, I1/Iγ, K, B/O	*aac(6')-Ib-cr*	bla_{TEM}
T_{AML}.h1fc53	AML			R	R	S	S	R	S	R	S	0.015	I1/Iγ, K	nd	bla_{TEM}
H1FC54		HE	ST93 (ST168 Cplx)	R	R	R	S	R	R	R	R	>32	FIA, FIB, FII, HI2	*aac(6')-Ib-cr*	bla_{OXA-1}, bla_{SHV}, bla_{TEM}
T_{AML}.h1fc54	AML			R	R	S	S	S	S	R	S	0.25	HI2	*aac(6')-Ib-cr*	bla_{SHV}
T_{CIP}.h1fc54	CIP			R	R	S	S	S	S	R	R	0.18	HI2	*aac(6')-Ib-cr*	bla_{SHV}, bla_{OXA-1}
H1PC19		HE	ST131 (ST131 Cplx)	R	R	R	R	R	R	R	R	>32	FIB, FII	*aac(6')-Ib-cr*	bla_{OXA-1}
T_{CIP}.h1pc19	CIP			S	S	S	S	S	S	S	S	12	nd	nd	nd
A1FcC2		RWW	ST224 (None)	R	R	S	S	R	S	R	S	>32	FIB, FII, I1/Iγ	*qnrS*, *aac(6')-Ib-cr*	bla_{OXA-1}, bla_{TEM}
T_{AML}.a1fcc2	AML			R	S	S	S	S	S	R	S	0.03	I1/Iγ	*qnrS*	bla_{TEM}
T_{CIP}.a1fcc2	CIP			R	S	S	S	S	S	S	S	0.12	nd	*qnrS*	bla_{TEM}
A2FcC14		RWW	ST131 (ST131 Cplx)	R	R	R	S	S	R	R	R	>32	FIA, FII, N	*aac(6')-Ib-cr*	bla_{CTX-M}, bla_{OXA-1}, bla_{TEM}
T_{AML}.a2fcc14	AML			R	S	S	S	S	R	R	R	0.015	FIA, FII, N	nd	bla_{TEM}
SA4FC71		RWW	ST1421 (None)	R	R	S	S	R	R	R	R	>32	FIB, FII, K, B/O	*qnrS*	bla_{TEM}
T_{AML}.sa4fc71	AML			R	S	S	S	S	S	R	S	0.25	K	*qnrS*	bla_{TEM}
T_{CIP}.sa4fc71	CIP			R	S	S	S	S	S	S	S	0.12	K, B/O	*qnrS*	bla_{TEM}

AML, amoxicillin; TIC, ticarcillin; CP, cephalotim, CAZ, ceftazidime; GEN, gentamycin; STR, streptomycin; SXT, sulfamethoxazole/trimethoprim; SUL, sulfamethoxazole; TET, tetracycline. All isolates were susceptible to meropenem and colistin; MDR, number of classes of antibiotics to which resistance was displayed. *donor strain *E.coli* J53 $MIC_{CIP} = 0.015\ \mu g\ mL^{-1}$. nd – not detected.

reduced susceptibility to fluoroquinolones, recovered from water environments over a period of nine years. These strains, selected from a collection of 776 isolates were characterized in terms of genetic diversity and prevailing mechanisms of quinolone resistance, aiming to infer about the potential for PMQR horizontal gene transfer. Isolates from different origins, dates and conditions of isolation clustered together. These results suggest that the same *E. coli* lineages are dispersed over water environments as distinct as HE, urban wastewater treatment plant raw inflow and treated effluent and urban streams. Most of the observed MLST are ubiquitous, being frequently reported in association with humans (in clinical context or in the community) or in animal husbandry. STs prevalent in this study, such as ST10, ST131, ST167 and ST57, have been reported in clinical isolates (Coque *et al.* 2008; Oteo *et al.* 2009, 2010, 2012; Cao *et al.* 2011; Poulsen *et al.* 2013; Guillard *et al.* 2014), in wastewater impacted aquatic environments (Amos *et al.* 2014) and also in pets and livestock (Giufrè *et al.* 2012; Shepard *et al.* 2012; Mora *et al.* 2013; Pires-dos-Santos, Bisgaard and Christensen 2013; Schink *et al.* 2013; Wagner, Gally and Argyle 2014). Other STs detected in wastewater, such as ST533, ST648 and ST744 have been reported in pets (Huber *et al.* 2013; Schink *et al.* 2013; Wagner, Gally and Argyle 2014), and ST93, ST101, ST155, ST224 and ST1421 in livestock (Hasan *et al.* 2012; Pires-dos-Santos, Bisgaard and Christensen 2013). This evidence suggests a possible circulation of the same lineages over different hosts and environmental compartments. The role of some of these lineages on antibiotic resistance propagation has been noted before, as clinical isolates are frequently associated to quinolone resistance (e.g. Jones *et al.* 2008) and extended-spectrum beta-lactamase genes (e.g. Mellmann *et al.* 2008; Valverde *et al.* 2009). In addition to antibiotic resistance, some of these lineages are also recognized by their virulence potential either in humans or livestock. Members of the ST131 and ST57, respectively, related with the phylogroups B2 and D, have been shown to carry a wide range of virulence genes associated with their MDR profiles (Johnson *et al.* 2010; Ben Sallem *et al.* 2012; Wagner, Gally and Argyle 2014). These evidences show that wastewater represents an important link in the circulation of bacteria of human and animal origin, although its role on the potential of transfer of these bacteria or resistance genes back to humans is still unclear.

In the present study, antibiotic resistance was found to be equally distributed among the different genetic lineages, suggesting the randomness of resistance acquisition. In contrast, the origin of isolates was determinant, with hospital and RWW representing major reservoirs of resistance, which suggests that HE may have a role as a source of emerging resistance types. Resistance to ceftazidime, a third-generation cephalosporin, which according to recent reviews, is still not among the most prevalent resistant types in wastewater environments (Rizzo *et al.* 2013; Vaz-Moreira, Nunes and Manaia 2014) was mainly observed in isolates from HE that were recovered on ciprofloxacin-supplemented culture medium. The coselection of quinolone and cephalosporin resistance phenotypes has been reported before for *Enterobacteriaceae* in clinical environment (Wener *et al.* 2010; Cao *et al.* 2011) and adds to the potential of bacteria to become MDR. In contrast, prevalence of tetracycline and MDR were lower among isolates from ciprofloxacin supplemented than from antibiotic-free culture medium. Although it may hint a bias imposed by the isolation procedure, this result suggests different paths of resistance acquisition for quinolones and tetracycline, as suggested in previous studies on the relationship between antibiotic residues and bacterial community variations (Novo *et al.* 2013; Varela *et al.* 2014).

Quinolone resistance is transmitted by both vertical and horizontal transfer processes, although, at least in *E. coli*, vertical transmission is certainly of great importance (Uchida *et al.* 2010; Figueira, Serra and Manaia 2011a; Shibl *et al.* 2012; Vredenburg *et al.* 2013). The importance of vertical transmission was evidenced also in this study. A particularly interesting evidence was given by group E ($n = 8$) in which all isolates, recovered over a period of six years and from distinct types of water presented the same Ile80(ATT) mutation, different from all the others. However, since chromosome mutations are apparently responsible for high MIC values, it is intriguing which kind of selective pressure may enhance horizontal gene transfer of PMQR or promote its selection. In this, as in previous studies, PMQR was preferentially found in bacteria isolated on ciprofloxacin-supplemented culture medium (Figueira *et al.* 2011b; Vredenburg *et al.* 2013). These observations suggest that PMQR may confer a selective advantage which is not necessarily related with an increased MIC value. Indeed, it was suggested that by conferring a low resistance level, PMQR may contribute to enhance the prevalence of resistant mutants in a population (Jacoby 2005). Accordingly, PMQR may act as facilitators for mutations acquisition, being lost in a subsequent stage, when they are no longer required. However, such an argument would not explain why PMQR was only detected among isolates from ciprofloxacin-supplemented culture medium. Moreover, the fact these determinants were only detected in raw effluents (from hospital and municipal treatment plants) may be an indication of a low stability in mobile genetic elements. On the other hand, while a certain instability may explain the low frequency observed, the association with distinct plasmid replicon types and bacterial lineages, suggests a wide distribution and independent circulation of the determinants *aac(6′)-Ib-cr* and *qnrS1*, also noted before (Park *et al.* 2006; Carattoli 2009; Guillard *et al.* 2014).

Through the analysis of distribution of PMQR over the distinct genetic lineages and associated plasmid replicons, it was expected to infer about the likelihood of horizontal gene transfer. The number of different replicon types was, in general, higher in isolates related with the STs ST10 complex and ST131, frequently reported in humans and animals and in clinical settings. Indeed, horizontal gene transfer are supposed to be favored in specific environments, preferentially directly subjected to anthropogenic impacts (Schlüter *et al.* 2007; Martinez 2009).

The ability of MDR bacteria to transfer their resistance determinants to a recipient strain was demonstrated *in vitro*. Supporting the phylogenetic inferences, the ability to conjugate was not related with the origin of the isolate, genetic lineage or plasmid replicon type. Three of the eight isolates harboring PMQR were able to transfer the genes *aac(6′)-Ib-cr* together with bla_{SHV} and bla_{OXA} (one isolate) or *qnrS1* together with bla_{TEM} (two isolates) and the plasmid replicon types I1-Iγ, K, B/O and/or HI2. These plasmid replicon types have been found in the environment, particularly in wastewater (Moura *et al.* 2012; Tacão *et al.* 2014), but also in poultry (Dierikx *et al.* 2010) and clinical samples (Valverde *et al.* 2009; Oteo *et al.* 2012), showing their widespread dissemination and suggesting their high potential to spread antibiotic resistance through different environmental compartments. Curiously, replicon types on the group IncF were not observed to be associated with PMQR transfer.

It is concluded that *E. coli* strains with reduced susceptibility to quinolones obtained from HE, streams and wastewater are genetically closely related, suggesting that the same populations can move freely among these and other environmental compartments. The concern here is that clinically relevant populations discharged from hospital and municipal effluents can reach the environment and persist in aquatic environments. Reduced susceptibility to quinolones was observed to be transferred through both vertical and horizontal gene transfer, mainly the genes *aac(6′)-Ib-cr* and *qnrS1* both demonstrated to be located on conjugative plasmids.

The results presented herein suggest that clinically relevant strains with complex antibiotic-resistant profiles that develop under the selective pressure of the hospital settings can be disseminated to the environment through wastewater discharges. Preventive measures, like separation of human excretions suspected of carrying emerging and/or high doses of antibiotic resistance and treatment of HE, could delay the global spread of such bacteria and their genetic determinants.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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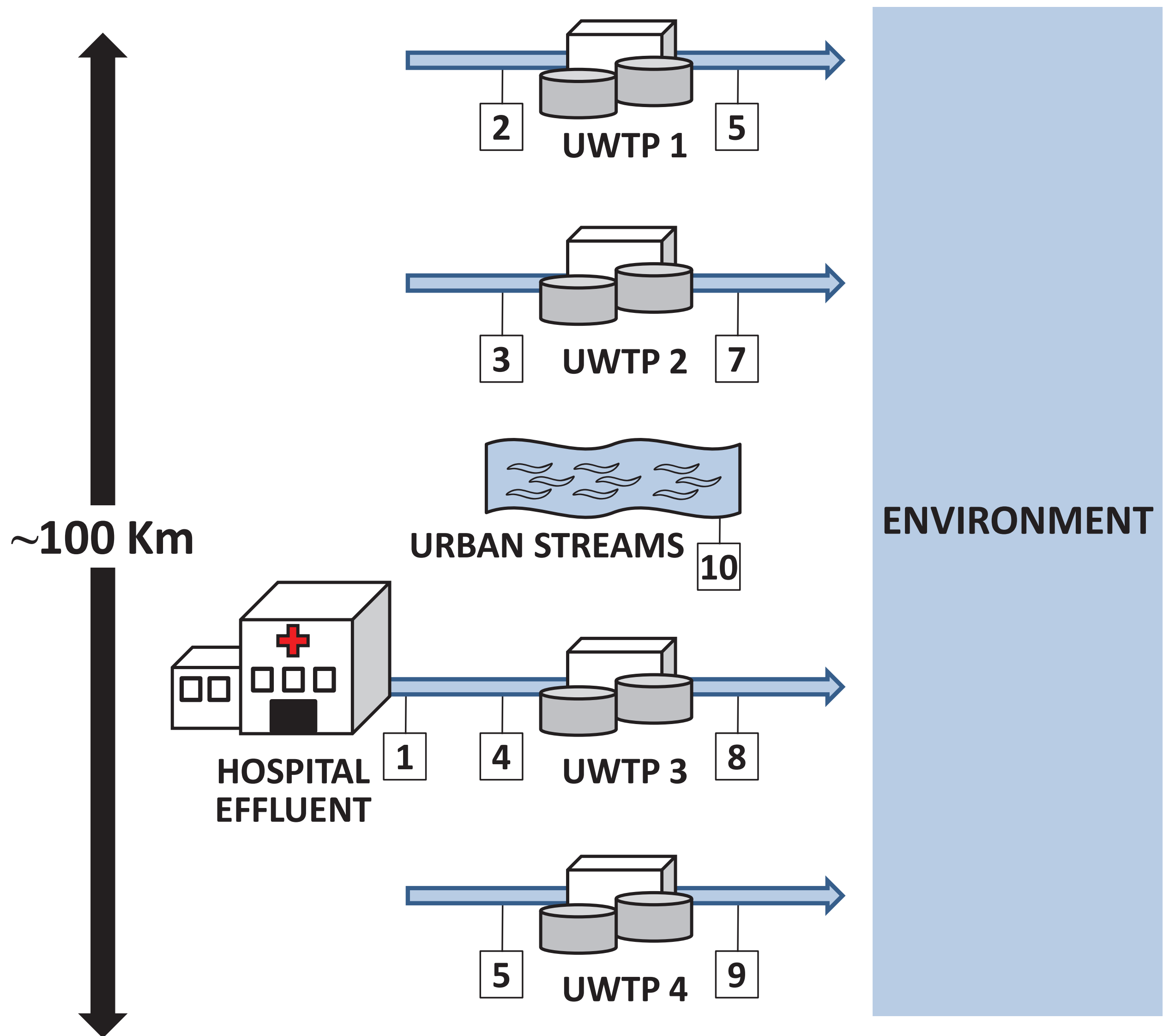

Figure S1 - Schematic representation of the sites of origin for the samples used in this study. UWTP, urban wastewater treatment plant; 1, raw hospital effluent; 2-5, raw inflow to the wastewater treatment plant; 6-9, treated effluent from the wastewater treatment plant; 10, urban stream; MDR>=3, resistance to three or more classes of antibiotics.

Chapter 3

Molecular evidence of the close relatedness of clinical, gull and wastewater isolates of quinolone-resistant *Escherichia coli*

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Short Communication

Molecular evidence of the close relatedness of clinical, gull and wastewater isolates of quinolone-resistant *Escherichia coli*

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ABSTRACT

Escherichia coli with reduced susceptibility to quinolones isolated from different environmental sources (urban wastewater treatment plants, $n = 61$; hospital effluent, $n = 10$; urban streams, $n = 9$; gulls, $n = 18$; birds of prey, $n = 17$) and from hospitalised patients ($n = 28$) were compared based on multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). The habitats with the most diversified genotypes of quinolone-resistant *E. coli*, corresponding to the highest genetic diversity (H'), were wastewater and gulls. In addition, genetically distinct populations were observed in clinical samples and birds of prey, suggesting the influence of the habitat or selective pressures on quinolone-resistant *E. coli*. The close genetic relatedness between isolates of clinical origin and from gulls and wastewater suggests the existence of potential routes of propagation between these sources. The most common sequence types were ST131 and ST10, with ST131 being highly specific to patients, although distributed in all of the other habitats except birds of prey. The prevalence of antimicrobial resistance was significantly higher in isolates from patients and gulls than from other sources ($P < 0.01$), suggesting that the effect of selective pressures met by isolates subjected to strong human impacts. The evidence presented suggests the potential circulation of bacteria between the environmental and clinical compartments, with gulls being a relevant vector of bacteria and resistance genes.
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1. Introduction

Originally regarded as a problem confined to clinical settings, nowadays antimicrobial resistance also concerns environmental health, herein referring to biological environmental factors that can potentially affect human health. In urban environments, wildlife, e.g. gulls, and wastewater treatment plants represent important reservoirs of antimicrobial-resistant bacteria and genetic determinants of resistance [1–4]. Wastewater, and in particular hospital effluents, are well known hotspots for the spread of resistance determinants occurring in clinical isolates, such as *vanA*, *aac(6′)-Ib*-cr and bla_{GES-5} [2,5].

Despite the increasing evidence that anthropogenic impacts are associated with the spread of antimicrobial resistance in the environment [3], the preferential routes of transmission of resistant bacteria from and to humans are still unclear. Molecular epidemiology-based studies can be used to track antimicrobial-resistant bacteria or genes in the environment [5]. The species *Escherichia coli*, which comprises environmental, human and other animal commensal strains, is able to survive in the environment and also includes opportunistic and multidrug-resistant (MDR) pathogens [6]. Therefore, *E. coli* constitutes an interesting model to assess the relationship between environmental and clinical antimicrobial resistance. In this study, we aimed to investigate whether the same *E. coli* lineages that are isolated from hospitalised patients can be found in a range of environmental settings. Quinolone-resistant *E. coli* isolated from gulls, birds of prey, urban streams, domestic raw and treated wastewater, and

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hospital effluent were compared with isolates from hospitalised patients based on multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE).

2. Methods

A subset of 115 *E. coli* strains was selected from a collection of ca. 600 environmental isolates based on their reduced susceptibility to quinolones (ciprofloxacin and/or nalidixic acid) as determined by the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [7]. Environmental isolates were recovered over a period of 9 years (2004–2012) in the northern region of Portugal from the following sources: raw inflow ($n = 28$) and treated effluent ($n = 33$) of four wastewater treatment plants; urban waterways ($n = 9$); untreated hospital effluent ($n = 10$); gulls ($n = 18$); and birds of prey (common buzzard, booted eagle, black kite, Eurasian black vulture, Eurasian tawny owl, Eurasian sparrow hawk, Bonelli's eagle and Eurasian eagle owl) ($n = 17$). Strains isolated from gulls and birds of prey were obtained from faecal samples collected within a distance of 200 km from the spots of water sampling. The environmental isolates were compared with consecutive and non-repetitive quinolone-susceptible [as determined by the disk diffusion method according to the Antibiogram Committee of the French Society for Microbiology (CA-SFM) from 2012; http://www.sfm-microbiologie.org] clinical isolates of *E. coli* ($n = 28$) from urine, fluids, blood, bronchoalveolar wash and swabs of patients admitted to a hospital in the northern region of Portugal over the year 2011 and sent to the National Reference Laboratory of Antibiotic Resistances and Healthcare Associated Infections at the National Institute of Health, in Lisbon, Portugal, as an integrative part of the laboratory Antimicrobial Resistance Surveillance Program in Portugal (ARSIP).

All isolates were typed by MLST using the housekeeping genes *adk* (536 bp), *fumC* (469 bp), *gyrB* (460 bp), *icd* (518 bp), *mdh* (452 bp), *purA* (478 bp) and *recA* (510 bp) as described by Wirth et al. [8]. The sequence type (ST) and ST complex were determined through http://mlst.warwick.ac.uk/mlst/dbs/Ecoli query as described previously [5]. A maximum parsimony tree based on the concatenated sequences of the seven MLST genes was built using BioNumerics v.6.6 (Applied Maths, Sint-Martens-Latem, Belgium) and its stability was assessed by other clustering methods (neighbour-joining and maximum likelihood). For three isolates it was not possible to obtain complete sequences for the *adk* gene. To avoid excluding these isolates, which shared 100% sequence similarity with others for the seven gene sequence fragments, the *adk* sequence used for the construction of the maximum parsimony tree had 449 positions, totalling 3336 nucleotide positions in the concatenated sequences.

PFGE performed as previously described using the restriction enzyme *Xba*I [9] was used to type isolates displaying the most prevalent sequence types (ST10 and ST131). A λ ladder pulsed-field gel marker (New England Biolabs, Hitchin, UK) was used as a size standard. Dice coefficients and the unweighted pair-group method (UPGMA) with the arithmetic averages clustering method were used for grouping analysis using BioNumerics v.6.6. Isolates with a Dice band-based similarity coefficient value of $\geq$80% were considered to belong to the same cluster. Isolates were tested for resistance to ticarcillin (TIC), ceftazidime (CAZ), meropenem (MEM), gentamicin (GEN) and trimethoprim/sulfamethoxazole (SXT). This was performed based on the disk diffusion method according to CLSI guidelines [7] for all environmental isolates and according to the CA-SFM (2012) for clinical isolates.

To elucidate the mechanisms of quinolone resistance, which was the main criterion for isolate selection, the presence of the plasmid-mediated quinolone resistance (PMQR) genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *oqxA*, *oqxB* and *aac(6′)-Ib*-cr as well as quinolone resistance-associated mutations in the genes *aac(6′)-Ib*-cr, *gyrA* and *parC* were assessed as described previously [5,10].

Isolates were compared regarding their genetic diversity based on the number of MLST sequence types found in each habitat, according to the diversity index (H') of Shannon and Weaver [11]. The evenness index was determined to compare the prevalence of different lineages in a given environmental source [11]. The prevalence of antimicrobial resistance was compared among isolates from different origins using Monte Carlo simulations of Fisher's exact test based on sampling 10 000 tables at a significance level (P-value) of $<$0.01. Statistical analyses were performed using SPSS Statistics for Windows v.19.0 (IBM Corp., Armonk, NY).

3. Results and discussion

MLST analysis allowed the matching of the 143 isolates with 54 different sequence types in the database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli), with ST131 (26 isolates), ST10 (9 isolates) and ST115 (8 isolates) being the most frequent types (Fig. 1). For three isolates (BR9, SG4 and SG8) it was not possible to obtain a complete sequence of the *adk* gene (Fig. 1). The corresponding tree comprised four major groups; group A with 30 isolates; group B with 45 isolates; group C with 45 isolates; and group D with 23 isolates. Clinical isolates were distributed over the four clusters, with 64.3% (from urine, swab, fluids, blood and bronchoalveolar wash) in group A, 21.4% (from urine) in group B, 10.7% (from urine and swab) in group C and 3.6% (from blood) in group D. The distribution of isolates from birds of prey, which inhabit mainly non-urban areas although with occasional association with anthropogenic environments, contrasted with that of clinical isolates. Isolates from birds of prey were not detected in clusters A and B, being distributed in groups C (47.1%) and D (52.9%). The ubiquity of the lineages colonising water and gulls was demonstrated in a homogeneous distribution of isolates in the different clusters, comprising 12.5% and 11.1% of cluster A, 41.3% and 33.3% of cluster B, 32.3% and 44.4% of cluster C, and 13.8% and 11.1% of cluster D, respectively. These data were consistent with the diversity indices observed in these habitats that could be ranked, from high to low diversity, as raw wastewater > treated wastewater > gulls > hospital effluent > urban streams > clinical > birds of prey (Table 1). Since wastewater, hospital effluent and urban streams receive *E. coli* from different sources, it is expected that they present high diversity indices compared with isolated habitats. This rationale puts also in evidence the intruder behaviour of gulls in urban areas. Indeed, the diversity index observed for gulls is a good indication of the diversity of sources of *E. coli* with which these birds may have contact and also hints at their potential as vectors of micro-organisms. The habitats with the lowest diversity indices were the patients and the birds of prey. The evenness index is a good indication of the potential of some lineages to prevail among others in a given habitat. Accordingly, the highest specificity was observed in birds of prey and patients (J values ranging from 0.47 to 0.51), mainly due to ST115 and ST131, respectively (Table 1). Although for birds of prey the evenness observed can be attributed to their isolation from human-impacted environments, in the case of clinical strains the lower diversity is more likely ascribed to the presence of selective pressures that can impose a bottleneck effect on the diversity of the population, as previously reported for human-associated bacterial communities [12].

Escherichia coli ST131, herein with high specificity to patients, is well known as an opportunistic pathogen with a widespread environmental distribution and MDR character [6]. The data presented in Fig. 1 confirm that ST131 can be propagated in the environment through water with faecal contamination, in

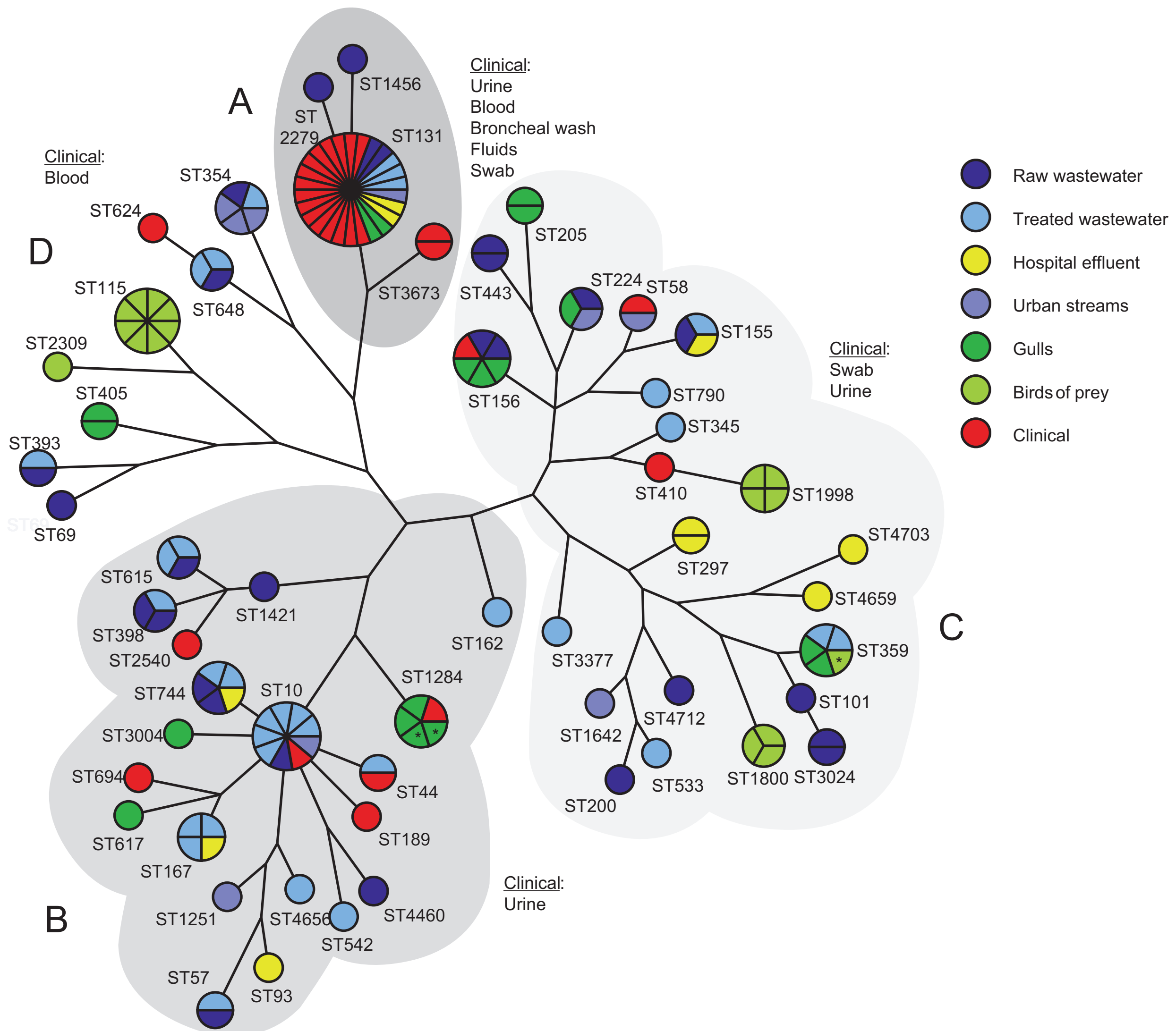

Fig. 1. Genetic relatedness of *Escherichia coli* isolates from hospital effluent, raw and treated wastewater from four urban wastewater treatment plants, gulls, birds of prey, urban streams and hospitalised patients, represented based on maximum parsimony tree of concatenated nucleotide sequences of the housekeeping genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* (number of positions = 3336), constructed with BioNumerics v.6.6 (Applied Maths, Sint-Martens-Latem, Belgium). ST, sequence type. Grey intensity, proportional to the prevalence of clinical isolates. * Isolates with short *adk* sequence.

particular hospital effluent, or gulls. By contrast, *E. coli* ST115, although found in water and gulls, was mainly associated with birds of prey. Although these results could suggest that ST115 is typical of low human-impacted sites, this genotype has been identified in hospital patients [13]. The interchangeability between niches was evident for other lineages, which were detected in patients and environmental sources, e.g. in gulls (ST156, ST1284), raw (ST10, ST156) and treated wastewater (ST10, ST44), and urban streams (ST10, ST58). The broad distribution of ST131 and ST10 detected in this study can be attributed to their high prevalence among the isolates. Nevertheless, the ubiquity of ST131 and ST10 has been demonstrated in previous studies [5,6]. PFGE typing confirmed that some strains can spread over different environmental compartments (Supplementary Fig. S1). The isolates of ST10 ($n = 7$) and ST131 ($n = 24$) whose clonal diversity was studied by PFGE yielded 16 PFGE profiles: 11 profiles included a single isolate and the other 5 profiles, defined as clusters I–V, included related ($\geq$80% similarity) or indistinguishable (100% homology) isolates (Supplementary Fig. S1). Cluster I included nine isolates from four origins (three isolated from patients, three from treated wastewater, two from raw wastewater and one from hospital effluent). Cluster V grouped ST10 isolates from raw and treated wastewater of different wastewater treatment plants, and cluster II grouped ST131 isolates from gulls and patient urine. The similarity between isolates from distinct locations suggests the existence of pathways of dissemination of MDR bacteria between the clinical settings and different environmental niches. Indeed, the potential of members of ST131 and ST10 to acquire and disseminate resistance across different environmental compartments has been evidenced in other studies with isolates recovered from birds or clinical isolates that display different resistance phenotypes [14].

Supplementary Fig. S1 related to this article can be found, in the online version, at doi:10.1016/j.jgar.2015.07.008.

Screening for quinolone resistance-associated mutations revealed that 92.3% ($n = 132$) of the isolates possessed mutations in the sequences of the genes *gyrA* (Leu83, $n = 8$; Ala83, $n = 1$; Asn87, $n = 1$; Leu83 Lys87, $n = 1$; Leu83 Asn87, $n = 108$; Leu83 Gly87, $n = 9$; and Leu83 Tyr87, $n = 3$) and/or *parC* (Ile80, $n = 96$; Arg80, $n = 4$; Arg80 Val84, $n = 1$; Ile80 Ala84, $n = 3$; Ile80 Gly84, $n = 3$; and Ile80 Val84, $n = 11$) (Supplementary Table S1).

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Table 1
Diversity and evenness indices based on the number of sequence types (STs) identified by multilocus sequence typing within each source.

Source	Isolates (*n*)	ST (*n*)	Diversity (*H*′)	Evenness (*J*)	%MDR4
Raw wastewater	28	22	3.01	0.90	32.1
Treated wastewater	33	20	2.79	0.81	15.2*
Hospital effluent	10	8	2.03	0.88	60.0
Urban streams	9	7	1.83	0.83	22.2
Gulls	18	9	2.09	0.72	66.7†
Birds of prey	17	5	1.33	0.47	0.0*
Clinical	28	12	1.70	0.51	64.3†

MDR4, resistance to at least one antimicrobial agent belonging to four or more classes of antibiotics.
*Significantly lower and † significantly higher ($P < 0.01$) than among isolates from other sources.

Of the nine PMQR genes analysed, only *aac(6′)-Ib*-cr ($n = 25$; 17.5% of the isolates), *qnrB* ($n = 3$; 2.1% of the isolates), *qnrS* ($n = 2$; 1.4% of the isolates) and *qepA* ($n = 2$; 1.4% of the isolates) were detected (Supplementary Table S1). The gene *aac(6′)-Ib*-cr was present in isolates from urban wastewater, hospital effluent, gulls, patient urine, blood and fluids, whilst *qepA* and *qnrS* were only detected in wastewater and *qnrB* in patient urine isolates. Although the presence of the PMQR genes *aac(6′)-Ib*-cr, *qnrS* and *qepA* is not indicative of high-level resistance phenotypes, as they are known to confer only low-level resistance to quinolones [15], it has been suggested that their occurrence can create a background of reduced susceptibility that may allow the emergence of higher-level resistance [15]. The detection of these genes in isolates of different origins and lineages in this study hints at their potential for horizontal transfer, which makes them an issue of concern when coupled with their role in the emergence of antimicrobial resistance.

Although the same bacterial lineages were found in distinct sources, the prevalence of MDR4 (resistance to antimicrobial agents of four or more classes) differed according to the origin of the isolates. A significantly higher number of MDR4 isolates ($P < 0.01$) was found among clinical isolates and urban gulls than among birds of prey (Table 1). These results likely reflect the high selective pressure experienced by clinical strains and the isolation of birds of prey from human activities. It is noticeable, however, that urban gulls are carriers of strains with resistance to multiple antimicrobial agents at levels that match that of clinical isolates.

In conclusion, it was possible to observe that closely related *E. coli* lineages can be found in hospitalised patients and isolated from environmental sources such as gulls, urban streams, domestic raw and treated wastewater, and hospital effluent. These findings suggest the existence of pathways of dissemination between these different environmental settings. The fact that the highest diversity of bacterial lineages was found in the subsets of isolates recovered from gulls and wastewater (including hospital effluent) is likely attributed to the high mobility that both gulls and wastewater have in the environment, which makes them prone to contact with a high range of sources of bacterial input. An additional consequence of this mobility is the potential to disseminate bacterial lineages in the environment. The results of this study suggest that the clinical settings and different environmental compartments may be considered communicating vessels through which bacteria and resistance genes are able to flow, and that wastewater and gulls may be significant vehicles of dissemination of antimicrobial resistance in the environment.

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Competing interests

None declared.

Ethical approval

Not required.

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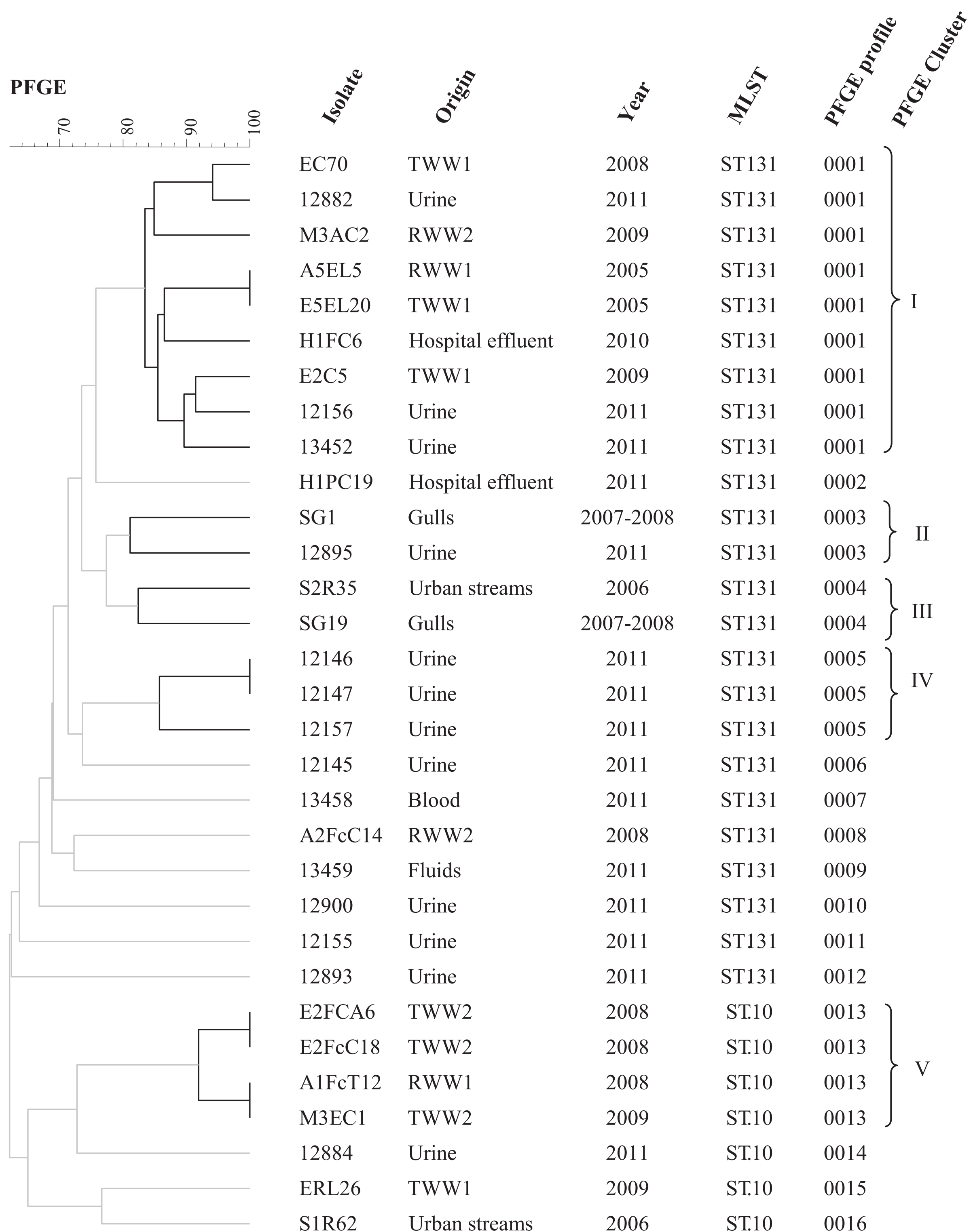

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Supplementary Fig. S1. Pulsed-field gel electrophoresis (PFGE) dendrogram and genetic relatedness of 31 of the most prevalent Escherichia coli sequence types (ST10 and ST131). Isolate, origin, year of isolation, multilocus sequence typing (MLST) and PFGE profile types (types 0001 to 0016 were defined as forming clusters I–V, respectively) are also shown. RWW1–2 and TWW1–2, raw inflow and treated effluent of the urban wastewater treatment plants 1–2, respectively.

Supplementary Table S1

Quinolone-resistant *Escherichia coli* isolates [a]: origin, antibiotic resistance pattern and presence of quinolone resistance genes

			Antibiotic resistance pattern [c]						QRDR		
Isolate	**Origin [b]**	**Year**	**TIC**	**CAZ**	**GEN**	**SXT**	**CIP**	**MDR [d]**	***parC***	***gyrA***	**PMQR genes**
A1FcC2	RWW1	2008	R	S	S	S	R	2	Ile80	Leu83 Lys87	*aac(6')-Ib*-cr, *qnrS*
A1FcT12	RWW1	2008	R	R	R	S	R	4	Ile80	Leu83 Asn87	
A2C1	RWW1	2009	R	R	S	S	R	3	Ile80	Leu83 Asn87	
A2FC9	RWW1	2004	R	S	S	R	R	3	Arg80	Leu83	
A2FcC14	RWW2	2008	R	R	S	R	R	4	Ile80	Leu83 Asn87	*aac(6')-Ib*-cr
A2P34	RWW1	2004	R	S	S	R	R	3	Ile80	Leu83 Asn87	
A3FcC1	RWW3	2008	S	S	S	R	R	2	Ile80	Leu83 Asn87	
A3FcT1	RWW3	2008	S	S	S	R	R	2	Ile80	Leu83 Asn87	
A4EL37	RWW1	2004	R	S	R	R	R	4	WT	WT	
A4FC15	RWW1	2004	R	S	S	R	R	3	WT	Leu83	
A4FC39	RWW1	2004	R	S	R	R	R	4	Ile80	Leu83 Asn87	
A5EL5	RWW1	2005	R	S	R	R	R	4	NA	Leu83 Asn87	
A6FC25	RWW1	2005	R	S	S	S	R	2	Arg80	Leu83 Asn87	
A6FC32	RWW1	2005	R	S	S	R	R	3	Ile80	Leu83 Asn87	
A6P59	RWW1	2005	R	S	S	S	R	2	Arg80	Leu83 Asn87	
A7P46	RWW1	2005	R	S	R	R	R	4	WT	WT	
AC104	RWW1	2009	R	S	S	S	R	2	Ile80	Leu83 Gly87	
AC111	RWW1	2009	R	S	R	S	R	3	Ile80	Leu83 Asn87	
ApCip1	RWW1	2009	R	S	S	R	R	3	Ile80 Gly84	Leu83 Asn87	
ApCip15	RWW1	2009	R	S	S	R	R	3	Ile80	Leu83 Asn87	

Isolate	Origin [b]	Year	Antibiotic resistance pattern [c]						QRDR		PMQR genes
			TIC	CAZ	GEN	SXT	CIP	MDR [d]	*parC*	*gyrA*	
E2C4	TWW1	2009	S	S	S	S	R	1	Ile80	Leu83 Asn87	
E2C5	TWW1	2009	S	S	S	S	R	1	NA	Leu83 Asn87	
E2FC17	TWW1	2004	R	S	S	R	R	3	Ile80	Leu83 Asn87	
E2FC23	TWW1	2004	R	S	R	S	R	3	Ile80	Leu83 Asn87	
E2FC26	TWW1	2004	R	S	S	R	R	3	WT	WT	
E2FCA6	TWW2	2008	R	R	R	S	R	4	Ile80	Leu83 Asn87	
E2FcC2	TWW2	2008	R	S	R	R	R	4	Ile80	Leu83 Asn87	
E2FcC3	TWW2	2008	R	S	S	S	R	2	Ile80	Leu83 Asn87	
E2FcC15	TWW2	2008	R	S	S	R	R	3	Ile80	Leu83 Asn87	
E2FcC16	TWW2	2008	R	S	S	R	R	3	Ile80	Leu83 Asn87	
E2FcC18	TWW2	2008	R	R	R	S	R	4	Ile80	Leu83 Asn87	
E3FC20	TWW1	2004	R	S	S	R	R	3	Ile80	Leu83 Asn87	
E3FC39	TWW1	2004	S	S	S	S	R	1	Ile80	Leu83 Asn87	
E3FC46	TWW1	2004	R	S	S	S	R	2	Ile80 Gly84	Leu83 Asn87	
E3FC53	TWW1	2004	R	S	S	R	R	3	Ile80	Leu83 Tyr87	
E4FC12	TWW1	2004	R	S	S	R	R	3	WT	Leu83	
E4FC22	TWW1	2004	R	S	S	R	R	3	Ile80	Leu83 Asn87	
E5EL20	TWW1	2005	R	S	R	R	R	4	NA	Leu83 Asn87	
E5FC25	TWW1	2005	R	S	S	R	R	3	Ile80	Leu83 Asn87	
E6FC24	TWW1	2005	R	S	S	S	R	2	Ile80	Leu83 Asn87	
EC46	TWW1	2008	S	S	S	R	R	2	Ile80	Leu83 Asn87	
EC70	TWW1	2008	R	S	S	R	R	3	Ile80 Val84	Leu83 Asn87	

			Antibiotic resistance pattern [c]						QRDR		
Isolate	**Origin [b]**	**Year**	**TIC**	**CAZ**	**GEN**	**SXT**	**CIP**	**MDR [d]**	***parC***	***gyrA***	**PMQR genes**
EC84	TWW1	2009	R	S	S	S	R	2	Ile80	Leu83 Asn87	
EC85	TWW1	2009	S	S	S	S	R	1	Ile80	Leu83 Asn87	
EC101	TWW1	2009	R	S	S	S	R	2	Ile80	Leu83 Asn87	
EFC3	TWW1	2008	R	S	S	R	R	3	Ile80	Leu83 Asn87	
EpCip2	TWW1	2009	R	S	S	R	R	3	Ile80	Leu83 Asn87	
ERL26	TWW1	2009	R	S	S	S	R	2	Ile80	Leu83 Asn87	
ERL45	TWW1	2009	R	S	S	S	R	2	Ile80	Leu83 Asn87	
ERL46	TWW1	2009	R	S	S	S	R	2	Ile80	Leu83 Asn87	
M1EC2	TWW2	2009	R	S	S	R	R	3	Ile80	Leu83 Asn87	
M2AC7	RWW2	2009	R	S	S	R	R	3	Ile80	Leu83 Asn87	
M2AC8	RWW2	2009	R	S	S	S	R	2	Ile80	Leu83 Asn87	
M2AC9	RWW2	2009	R	S	R	R	R	4	Ile80	Leu83 Asn87	
M3AC1	RWW2	2009	R	S	S	R	R	3	Ile80	Leu83 Asn87	
M3AC2	RWW2	2009	S	S	S	S	R	1	Ile80	Leu83 Asn87	
M3EC1	TWW2	2009	R	R	R	S	R	4	Ile80	Leu83 Asn87	
M3EC2	TWW2	2009	R	S	S	S	R	2	Ile80	Leu83 Asn87	
S1R20	Urban streams	2006	R	S	S	R	R	3	Arg80	Leu83	
S1R62	Urban streams	2006	R	S	R	R	R	4	WT	Leu83	
S2R35	Urban streams	2006	R	S	S	R	R	3	NA	Asn87	
S3R22	Urban streams	2006	R	S	S	R	R	3	Ile80	Leu83 Asn87	
S3R47	Urban streams	2006	S	S	R	S	R	2	Ile80	Leu83 Asn87	
S3R50	Urban streams	2006	S	S	R	S	R	2	Ile80	Leu83 Asn87	

			Antibiotic resistance pattern [c]						QRDR		
Isolate	**Origin [b]**	**Year**	**TIC**	**CAZ**	**GEN**	**SXT**	**CIP**	**MDR [d]**	***parC***	***gyrA***	**PMQR genes**
H1PC4	Hospital effluent	2010	R	R	R	R	R	**5**	Ile80	Leu83 Asn87	
H1FC5	Hospital effluent	2010	R	R	S	R	R	**4**	Ile80	Leu83 Asn87	
H1FC6	Hospital effluent	2010	R	S	S	R	R	**3**	NA	Leu83 Asn87	
H1FC21	Hospital effluent	2011	R	R	S	R	R	**4**	Ile80	Leu83 Asn87	
H1FC24	Hospital effluent	2011	R	S	S	R	R	**3**	Ile80	Leu83 Asn87	
H1FC29	Hospital effluent	2011	S	S	R	S	R	**2**	Ile80	Leu83 Asn87	
H1FC30	Hospital effluent	2011	R	S	S	S	R	**2**	Ile80	Leu83 Asn87	
H1FC53	Hospital effluent	2011	R	R	S	R	R	**4**	Ile80	Leu83 Asn87	*aac(6')-Ib*-cr
H1FC54	Hospital effluent	2011	R	R	S	R	R	**4**	Ile80	Leu83 Asn87	*aac(6')-Ib*-cr
H1PC19	Hospital effluent	2011	R	R	R	R	R	**5**	NA	Leu83 Asn87	*aac(6')-Ib*-cr
SA4FC36A	RWW4	2010	R	S	R	R	R	**4**	Ile80	Leu83 Asn87	*qepA*
SA4FC71	RWW4	2011	R	S	S	R	R	**3**	Ile80	Leu83 Asn87	*qnrS*
SA4FC77	RWW4	2011	R	R	R	S	R	**4**	Ile80	Leu83 Asn87	*qepA*
CC4	Urban streams	2012	R	S	S	S	R	**2**	Ile80	Leu83 Asn87	
CC5	Urban streams	2012	S	S	S	R	R	**2**	Ile80	Leu83 Asn87	
DA1	Urban streams	2012	R	S	R	R	R	**4**	Ile80 Gly84	Leu83 Asn87	
BR1	Birds of prey (Common buzzard)	2008	R	S	S	R	R	**3**	Ile80	Leu83 Asn87	
BR10	Birds of prey (Eurasian black vulture)	2008	R	S	S	R	R	**3**	Ile80	Leu83 Gly87	
BR11	Birds of prey (Eurasian black vulture)	2008	R	S	S	R	R	**3**	Ile80	Leu83 Gly87	
BR12	Birds of prey (Eurasian black vulture)	2008	R	S	S	R	R	**3**	Ile80	Leu83 Gly87	
BR13	Birds of prey (Eurasian tawny owl)	2008	R	S	S	R	R	**3**	Ile80	Leu83 Tyr87	
BR15	Birds of prey (Eurasian sparrow hawk)	2008	R	S	S	R	R	**3**	Ile80	Leu83 Gly87	

Isolate	Origin [b]	Year	Antibiotic resistance pattern [c]						QRDR		PMQR genes
			TIC	CAZ	GEN	SXT	CIP	MDR [d]	*parC*	*gyrA*	
BR16	Birds of prey (Common buzzard)	2008	**R**	**S**	**S**	**R**	**R**	**3**	Ile80	Leu83 Gly87	
BR17	Birds of prey (Bonelli's eagle)	2008	**R**	**S**	**S**	**R**	**R**	**3**	Ile80	Leu83 Gly87	
BR18	Birds of prey (Eurasian eagle owl)	2008	**R**	**S**	**S**	**R**	**R**	**3**	Ile80	Leu83 Gly87	
BR19	Birds of prey (Eurasian eagle owl)	2008	**R**	**S**	**S**	**R**	**R**	**3**	Ile80	Leu83 Gly87	
BR3	Birds of prey (Common buzzard)	2008	**R**	**S**	**S**	**R**	**R**	**3**	Ile80	Leu83 Asn87	
BR4	Birds of prey (Booted eagle)	2008	**R**	**S**	**S**	**R**	**R**	**3**	Ile80	Leu83 Asn87	
BR5	Birds of prey (Common buzzard)	2008	**R**	**S**	**S**	**R**	**R**	**3**	Ile80	Leu83 Asn87	
BR6	Birds of prey (Common buzzard)	2008	**R**	**S**	**S**	**R**	**R**	**3**	Ile80	Leu83 Asn87	
BR7	Birds of prey (Booted eagle)	2008	**R**	**S**	**S**	**R**	**R**	**3**	Ile80	Leu83 Asn87	
BR8	Birds of prey (Booted eagle)	2008	**R**	**S**	**S**	**R**	**R**	**3**	Ile80	Leu83 Asn87	
BR9	Birds of prey (Black kite)	2008	**R**	**S**	**S**	**R**	**R**	**3**	Ile80	Leu83 Asn87	
SG1	Gulls	2007-2008	**R**	**R**	**R**	**S**	**R**	**4**	NA	Leu83 Asn87	
SG10	Gulls	2007-2008	**R**	**R**	**S**	**R**	**R**	**4**	Ile80	Leu83 Asn87	*aac(6')-Ib*-cr
SG11	Gulls	2007-2008	**R**	**R**	**R**	**R**	**R**	**5**	Ile80	Leu83 Asn87	*aac(6')-Ib*-cr
SG12	Gulls	2007-2008	**R**	**S**	**R**	**S**	**R**	**3**	Ile80	Leu83 Asn87	
SG14a	Gulls	2007-2008	**R**	**R**	**S**	**R**	**R**	**4**	Ile80	Leu83 Asn87	*aac(6')-Ib*-cr
SG15	Gulls	2007-2008	**R**	**R**	**S**	**S**	**R**	**3**	Ile80 Ala84	Leu83 Asn87	
SG16	Gulls	2007-2008	**R**	**R**	**R**	**S**	**R**	**4**	Ile80 Ala84	Leu83 Asn87	
SG17	Gulls	2007-2008	**R**	**S**	**R**	**R**	**R**	**4**	Ile80	Leu83 Asn87	
SG18	Gulls	2007-2008	**R**	**R**	**R**	**R**	**R**	**5**	Ile80	Leu83 Asn87	*aac(6')-Ib*-cr
SG19	Gulls	2007-2008	**R**	**R**	**R**	**R**	**R**	**5**	Ile80 Val84	Leu83 Asn87	*aac(6')-Ib*-cr
SG2	Gulls	2007-2008	**R**	**S**	**S**	**R**	**R**	**3**	Arg80 Val84	Leu83 Tyr87	

			Antibiotic resistance pattern [c]						QRDR		
Isolate	Origin [b]	Year	TIC	CAZ	GEN	SXT	CIP	MDR [d]	*parC*	*gyrA*	PMQR genes
SG20	Gulls	2007-2008	**R**	**R**	**S**	**R**	**R**	**4**	Ile80	Leu83 Asn87	
SG3	Gulls	2007-2008	**R**	**R**	**R**	**R**	**R**	**5**	Ile80	Leu83 Asn87	
SG4	Gulls	2007-2008	**R**	**R**	**R**	**R**	**R**	**5**	Ile80	Leu83 Asn87	*aac(6')-Ib*-cr
SG5	Gulls	2007-2008	**R**	**S**	**S**	**R**	**R**	**3**	Ile80	Leu83 Asn87	
SG6	Gulls	2007-2008	**R**	**S**	**S**	**S**	**R**	**2**	Ile80	Leu83 Asn87	
SG7	Gulls	2007-2008	**R**	**S**	**S**	**R**	**R**	**3**	Ile80	Leu83 Asn87	
SG8	Gulls	2007-2008	**R**	**R**	**R**	**R**	**R**	**5**	Ile80	Leu83 Asn87	*aac(6')-Ib*-cr
12145	Urine	2011	**R**	**R**	**R**	**S**	**R**	**4**	Ile80 Val84	Leu83 Asn87	*aac(6')-Ib*-cr, *qnrB*
12146	Urine	2011	**R**	**R**	**R**	**S**	**R**	**4**	Ile80 Val84	Leu83 Asn87	*aac(6')-Ib*-cr, *qnrB*
12147	Urine	2011	**R**	**R**	**R**	**S**	**R**	**4**	Ile80 Val84	Leu83 Asn87	*aac(6')-Ib*-cr, *qnrB*
12148	Urine	2011	**R**	**R**	**S**	**R**	**R**	**4**	Ile80	Leu83 Asn87	
12151	Urine	2011	**R**	**R**	**R**	**R**	**R**	**5**	Ile80	Leu83 Asn87	*aac(6')-Ib*-cr
12155	Urine	2011	**R**	**R**	**R**	**R**	**R**	**5**	Ile80 Val84	Leu83 Asn87	*aac(6')-Ib*-cr
12156	Urine	2011	**R**	**R**	**S**	**S**	**R**	**3**	Ile80 Val84	Leu83 Asn87	*aac(6')-Ib*-cr
12157	Urine	2011	**R**	**R**	**R**	**R**	**R**	**5**	Ile80 Val84	Leu83 Asn87	
12158	Urine	2011	**R**	**R**	**S**	**R**	**R**	**4**	Ile80 Val84	NA	*aac(6')-Ib*-cr
12367	Swab	2011	**R**	**R**	**S**	**R**	**R**	**4**	NA	NA	
12370	Fluids	2011	**R**	**R**	**R**	**R**	**R**	**5**	NA	NA	NA
12882	Urine	2011	**R**	**R**	**S**	**R**	**R**	**4**	Ile80 Val84	Leu83 Asn87	
12883	Urine	2011	**R**	**S**	**S**	**S**	**S**	**1**	WT	Ala83	*aac(6')-Ib*-cr
12884	Urine	2011	**R**	**S**	**S**	**R**	**R**	**3**	Ile80	Leu83 Asn87	
12887	Fluids	2011	**R**	**S**	**S**	**S**	**S**	**1**	WT	NA	*aac(6')-Ib*-cr

			Antibiotic resistance pattern [c]						QRDR		
Isolate	**Origin [b]**	**Year**	**TIC**	**CAZ**	**GEN**	**SXT**	**CIP**	**MDR [d]**	***parC***	***gyrA***	**PMQR genes**
12888	Blood	2011	R	R	S	R	R	4	NA	NA	*aac(6')-Ib*-cr
12889	Urine	2011	R	R	S	R	R	4	Ile80 Ala84	Leu83 Asn87	
12890	Urine	2011	R	R	R	R	R	5	Ile80	Leu83 Asn87	
12892	Swab	2011	R	R	R	S	R	4	NA	NA	NA
12893	Urine	2011	R	R	S	S	R	3	WT	WT	*aac(6')-Ib*-cr
12895	Urine	2011	R	R	R	S	R	4	Ile80 Val84	Leu83 Asn87	*aac(6')-Ib*-cr
12897	Urine	2011	R	S	S	R	R	3	Ile80	Leu83 Asn87	
12900	Urine	2011	R	S	S	S	S	1	WT	Leu83	*aac(6')-Ib*-cr
13447	Bronchoalveolar wash	2011	R	R	S	S	R	3	NA	NA	NA
13451	Swab	2011	R	S	S	R	S	2	NA	NA	NA
13452	Urine	2011	R	R	S	S	R	3	Ile80	Leu83 Asn87	
13458	Blood	2011	R	R	R	R	S	4	WT	Leu83	
13459	Fluids	2011	R	R	R	R	S	4	WT	Leu83	

TIC, ticarcillin; CAZ, ceftazidime; GEN, gentamicin; SXT, trimethoprim/sulfamethoxazole; CIP, ciprofloxacin; MDR, multidrug-resistant; QRDR, quinolone resistance-determining region; PMQR, plasmid-mediated quinolone resistance, S, susceptible; R, resistant; WT, wild-type; NA, no data available.

[a] All isolates with reduced susceptibility to quinolones (ciprofloxacin and/or nalidixic acid).

[b] RWW1–4 and TWW1–2, raw inflow and treated effluent of the urban wastewater treatment plants 1 to 4, respectively.

[c] All isolates were susceptible to meropenem.

[d] Number of classes of antibiotics to which resistance was detected.

Chapter 4

Quinolone resistant *Aeromonas* spp. as carriers and potential tracers of acquired antibiotic resistance in hospital and municipal wastewater

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Quinolone resistant *Aeromonas* spp. as carriers and potential tracers of acquired antibiotic resistance in hospital and municipal wastewater

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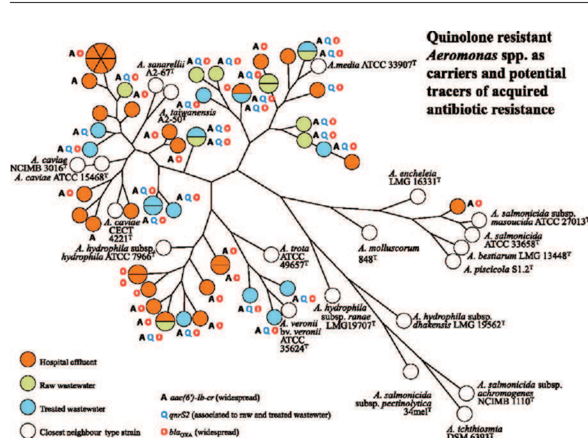
HIGHLIGHTS

- Characterization of quinolone resistant *Aeromonas* from urban wastewaters
- The highest prevalence of multidrug resistance among isolates from hospital effluent
- *Aeromonas* carry different antibiotic resistance genes depending on their origin.
- First report of the beta-lactamase gene *bla*$_{OXA-101}$ in *Aeromonas*

GRAPHICAL ABSTRACT



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ABSTRACT

Members of the genus *Aeromonas* are recognized carriers of antibiotic resistance in aquatic environments. However, their importance on the spread of resistance from hospital effluents to the environment is poorly understood. Quinolone resistant *Aeromonas* spp. (n = 112) isolated from hospital effluent (HE) and from raw (RWW) and treated wastewater (TWW) of the receiving urban wastewater treatment plant (UWTP) were characterized. Species identification and genetic intraspecies diversity were assessed based on the 16S rRNA, *cpn60* and *gyrB* genes sequence analysis. The antibiotic resistance phenotypes and genotypes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qnrVC*; *qepA*; *oqxAB*; *aac*(6′)*-Ib-cr*; *bla*$_{OXA}$; incU) were analyzed in function of the origin and taxonomic group. Most isolates belonged to the species *Aeromonas caviae* and *Aeromonas hydrophila* (50% and 41%, respectively). The quinolone and the beta-lactamase resistance genes *aac*(6′)*-Ib-cr* and *bla*$_{OXA}$, including gene *bla*$_{OXA-101}$, identified for the first time in *Aeromonas* spp., were detected in 58% and 56% of the isolates, respectively, with identical prevalence in HE and UWTP wastewater. In contrast, the gene *qnrS2* was observed mainly in isolates from the UWTP (51%) and rarely in HE isolates (3%), suggesting that its origin is not the clinical setting.

Bacterial groups and genes that allow the identification of major routes of antibiotic resistance dissemination are valuable tools to control this problem. In this study, it was concluded that members of the genus *Aeromonas* harboring the genes *aac*(6′)*-Ib-cr* and *bla*$_{OXA}$ are relevant tracers of antibiotic resistance dissemination in wastewater habitats, while those yielding the gene *qnrS2* allow the traceability from non-clinical sources.

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1. Introduction

Over the last years, antibiotic resistance in commensal and pathogenic bacteria has become an issue of growing concern, with the clinical settings representing only the top of the iceberg (World Health Organization, 2014). Among the multiple reservoirs and paths of antibiotic resistance dissemination, hospital, municipal sewage collectors and wastewater treatment plants are known to play an important role (Berendonk et al., 2015; Rizzo et al., 2013; Vaz-Moreira et al., 2014). *Aeromonas* spp. are ubiquitous bacteria, typical inhabitants of aquatic environments that may occur also in soils, animals or other environments (Janda and Abbott, 2010). As opportunistic pathogens, *Aeromonas* spp. have been implied in a number of infections in humans such as bacteremia or gastroenteritis (Piotrowska and Popowska, 2014).

It has been documented in different studies that members of the genus *Aeromonas* can harbor genes encoding beta-lactam and plasmid mediated quinolone resistance (PMQR), with the potential to spread via horizontal gene transfer (Cattoir et al., 2008a; Moura et al., 2012). The role of members of this genus on the dissemination of antibiotic resistance genes is related with the recognized genomic plasticity and ecology, with habitats including water sources associated with human activities (Abbott et al., 2003). While several studies have focused on the role of *Aeromonas* spp. as a reservoir of antibiotic resistance in water environments (Figueira et al., 2011; Tacão et al., 2014), the contribution of hospital effluents for the dissemination of these bacteria and their antibiotic resistance determinants remains unclear. Thus, the aim of the present study was to assess the role of *Aeromonas* spp. on the dissemination of antibiotic resistance. In particular, it was aimed to elucidate if the hospital effluent could be considered its major reservoir or if, in contrast, *Aeromonas* spp. could be indicators of antibiotic resistance from non-clinical sources. Since quinolone resistance is widespread in the environment and in clinical settings and significantly more prevalent in hospital effluent than in municipal wastewater (Parker and Shaw, 2011; Piotrowska and Popowska, 2014; Poirel et al., 2012; Varela et al., 2015a), this resistance phenotype was selected in this study to assess potential routes of dissemination of antibiotic resistance in aquatic environments. Hence, a set of quinolone (in this particular case nalidixic acid) resistant *Aeromonas* spp. isolated from hospital effluent and from the receiving wastewater treatment plant were selected as antibiotic resistance tags and compared for their genotypic diversity and antibiotic resistance profiles.

2. Materials and methods

2.1. Bacterial isolates

The study compared 112 nalidixic acid-resistant *Aeromonas* strains isolated from hospital effluent, raw inflow and treated effluent of the receiving urban wastewater treatment plant (UWTP). This UWTP also collects domestic sewage, pre-treated industrial effluents and untreated effluents from different healthcare facilities, and serves a population equivalent of 200,000 inhabitants. The strains were isolated during a study that analyzed seven samples of hospital effluent, 21 of the raw and 21 of treated effluent of the UWTP and that involved both culture-dependent and culture-independent methods (Varela et al., 2014). As part of culture-dependent methods, the prevalence of antibiotic resistance in the three types of water was compared for different bacterial groups, one of which was the aeromonads. Besides enumeration, bacteria were isolated aiming at studying epidemiological and antibiotic resistance patterns. Therefore, isolates recovered on different culture media were identified and organized into groups adequate to study either epidemiological aspects or the resistance genes (Varela et al., 2015a, 2015b). One of such groups, whose study is herein reported, was of nalidixic acid resistant *Aeromonas* spp., specifically 33 from untreated hospital effluent (HE), and 43 from raw (RWW) and 46 from treated wastewater (TWW) of the UWTP. These isolates were recovered on Glutamate Starch Phenol-red agar (GSP, Merck) supplemented with 4 mg/L ciprofloxacin (n = 74) or with 32 mg/L amoxicillin (n = 11), on m-Fecal Coliform agar (mFC, Difco) supplemented with 4 mg/L ciprofloxacin (n = 19) or on Plate Count Agar (PCA, Pronadisa) supplemented with 4 mg/L ciprofloxacin (n = 8).

2.2. Identification and genotyping

The isolates were identified based on 16S rRNA gene sequence analysis as described before (Ferreira da Silva et al., 2007) and EzTaxon database query (Kim et al., 2012). To evaluate possible sources and routes of dissemination of *Aeromonas* spp. harboring the quinolone resistance genes *aac(6′)-Ib-cr* and/or *qnrS*, intraspecific genetic variation was assessed based on the analysis of additional housekeeping genes. Partial sequences of the genes 16S rRNA (1323 bp), *cpn60* (555 bp) and *gyrB* (810 bp) were compared with the most similar sequences available in the GenBank (http://www.ncbi.nlm.nih.gov/). Concatenated sequences of the three gene fragments, in a total of 2688 bp (Ferreira da Silva et al., 2007; Miñana-Galbis et al., 2009; Yáñez et al., 2003), were aligned using ClustalW from MEGA 6.0 software, nucleotide sequence relatedness was estimated based on the model of Jukes and Cantor (Jukes and Cantor, 1969) and a dendrogram was created using the Neighbor-Joining method (Tamura et al., 2013). The maximum likelihood method was also applied to assess tree stability, using Bionumerics software (version 6.1, Applied Maths).

2.3. Antibiotic resistance phenotypes

The susceptibility to nalidixic acid (NA, 30 μg); ciprofloxacin (CIP, 5 μg); amoxicillin (AML, 25 μg); ticarcillin (TIC, 75 μg); cephalothin (CP, 30 μg); ceftazidime (CAZ, 30 μg); streptomycin (STR, 10 μg); sulfamethoxazole/trimethoprim (SXT, 25 μg); tetracycline (TET, 30 μg); gentamicin (GEN, 10 μg); colistin sulfate (CT, 50 μg) and meropenem (MEM, 10 μg) was determined based on the agar diffusion method (CLSI M100-S21, 2012). For the antibiotics AML and CT, which are not included in the CLSI list, the following criteria were used: S > 21/R < 14 and S > 10/R < 10, respectively. Inhibition zones larger than R and smaller than S were classified as intermediary resistance and excluded from the resistance percentage calculations. The strains *Escherichia coli* DSM 1103 (ATCC 25922) and *Pseudomonas aeruginosa* DSM 1117 (ATCC 27853) were included as quality controls.

2.4. Antibiotic resistance and related genetic determinants

The isolates were screened for the presence of the genes of plasmid mediated quinolone resistance (PMQR) (1. *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6′)-Ib-cr*, *qepA*; 2. *qnrVC*; 3. *oqxAB*) using the primers and conditions described in previous works, respectively (1, Vredenburg et al., 2014; 2, Tacão et al., 2014; 3, Liu et al., 2011). The presence of selected beta-lactam resistance genes ($bla_{OXA-1-4}$, bla_{OXA-7}, $bla_{OXA-10-11}$, $bla_{OXA-13-17}$, bla_{OXA-19}, bla_{OXA-21}, bla_{OXA-28}, $bla_{OXA-30-32}$ and $bla_{OXA-34-35}$), previously reported in water environments, was tested using the conditions described by Henriques et al. (2006). Sequences representative of the PMQR and bla_{OXA} amplicons were deposited in the GenBank (LN824094-LN824130).

The presence of the plasmid replicons types IncA/C, IncP, IncU and IncQ was tested using the primers and conditions previously described (Carattoli et al., 2005; Cattoir et al., 2008b; Götz et al., 1996). Class 1 integrons variable regions were screened for as described in Ferreira da Silva et al. (2007), and amplicons larger than 1000 bp were further analyzed for their nucleotide sequences, after GenBank database querying.

The following strains were included in the respective PCR reactions as positive controls: *E. coli* L0 (*qnrA1*+); *Klebsiella pneumoniae* B1 (*qnrB1*+), *Enterobacter cloacae* S1 (*qnrS1*+) (Cattoir et al., 2007), *E. coli* TOP10+ paT851 (*qepA*+) (Périchon et al., 2007); *E. coli* DH10B

transformant pHS11 (*qnrC*+) (Wang et al., 2009); *E. coli* DH10B transformant p2007057 (*qnrD*+) (Cavaco et al., 2009); and bla_{OXA}+, *oqxA*+, *oqxB*+, *int1*+, *qnrVC*+ and incQ+ isolates from the laboratory's culture collection. The *qnrVC*+ and incQ+ control strains were clones of the strain *E. coli* JM109 transformed with amplicons of the genes *qnrVC* or incQ obtained from raw wastewater total DNA extracts, inserted in the plasmid pTZ57R/T using the InsTAclone™ PCR Cloning Kit (ThermoScientific). The respective nucleotide sequences were deposited in the GenBank under the accession numbers LN736033 and LN824131. Besides the use of positive controls, the authenticity of the PCR amplicons was confirmed based on nucleotide sequence analysis and query of the GenBank database using the BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Conjugation experiments

Nine isolates, representative of all types of water studied and harboring PMQR and/or beta-lactamase genes, were tested for their capacity to conjugate and transfer resistance traits to the sodium azide resistant *E. coli* J53 recipient strain. Transconjugants were selected on Luria–Bertani agar plates (LA) supplemented with azide (100 mg/L) and ciprofloxacin (0.06 mg/L) or amoxicillin (32 mg/L). Transconjugant identity was confirmed through random amplified polymorphic DNA genotyping, using the primers and conditions described elsewhere (Ferreira da Silva et al., 2007). Transconjugants were characterized based on acquired antibiotic resistance phenotype and genotype as described above. When necessary, the minimum inhibitory concentration for ciprofloxacin was determined using M.I.C. Evaluator strips (Oxoid), according to the manufacturer's instructions.

2.6. Statistical analyses

Statistical analyses were performed using SPSS 22.0 for Windows (SPSS Inc., Chicago, IL). The prevalence of antibiotic resistance phenotypes, antibiotic resistance genes or plasmid replicon types was compared among isolates from different origins or taxonomic groups using the chi-square test or Fisher's exact test at a significance level (α) of 0.05.

3. Results

3.1. Species diversity

A total of 112 isolates identified as members of the genus *Aeromonas* and with a phenotype of resistance to nalidixic acid were available for this study, 33 from hospital effluent, 43 from raw inflow and 36 from treated effluent of the wastewater treatment plant. Based on the 16S rRNA gene sequence analysis, the isolates could be classified into five taxonomic groups, the *Aeromonas allosaccharophila* group ($n = 5$), the *Aeromonas caviae/punctata* group ($n = 56$), the *Aeromonas hydrophila* group ($n = 46$), the *Aeromonas salmonicida* group ($n = 2$) and the *Aeromonas veronii* group ($n = 3$) (Tables S1 and S2). The abundance of members of each taxonomic group was not influenced by the culture medium used for isolation ($p > 0.05$). Members of the groups *A. caviae/punctata* and *A. hydrophila* were detected in the three types of water sampled and were the most prevalent, constituting 50% and 41% of all isolates, respectively. Members of the other groups represented less than 10% of the isolates and were found only in some types of water, specifically *A. salmonicida* (only in HE and RWW), *A. allosaccharophila* (only in RWW and TWW) and *A. veronii* (only in TWW) (Table S2).

3.2. Antibiotic resistance phenotypes

The quinolone resistant isolates examined in this study presented a high prevalence of multidrug resistance (93%, $n = 104$), i.e. resistance to antibiotics belonging to three or more different classes (MDR ≥ 3). The highest resistance prevalence values (>80%) were observed for the beta-lactams amoxicillin, ticarcillin or cephalothin, probably due to intrinsic resistance, at least in some *Aeromonas* species (Table 1). Resistance to ceftazidime, meropenem or tetracycline presented comparatively lower prevalence values (<20%) than to the other antibiotics tested, and isolates with these phenotypes were mainly from HE. Resistance to colistin was detected in a single HE isolate. In addition, isolates from HE presented significantly higher ($p < 0.05$) prevalence of resistance to ticarcillin, cephalothin, ceftazidime, gentamicin, streptomycin and sulfamethoxazole/trimethoprim, and significantly higher frequency of ($p < 0.05$) MDR ≥ 4 phenotypes (resistance to at least one antibiotic of four or more distinct classes of antibiotics) than those from the urban wastewater treatment plant. For most antibiotic classes, resistance prevalence was not significantly different ($p > 0.05$) among the distinct taxonomic groups. Exceptions were the significantly ($p < 0.05$) higher resistance prevalence to sulfamethoxazole/trimethoprim in *A. caviae/punctata* and to ticarcillin and cephalothin in *A. hydrophila*. Ceftazidime resistance was significantly more frequent ($p < 0.05$) among the isolates recovered on PCA with 4 mg/L of ciprofloxacin than on any other isolation culture medium.

3.3. Genetic determinants associated with antibiotic resistance

Of the nine PMQR genes analyzed, only the *aac(6′)-Ib-cr*, found in 59% of the isolates ($n = 66$) and *qnrS2*, observed in 37% of the isolates ($n = 41$), were detected in the *Aeromonas* spp. studied. The presence of both genes *aac(6′)-Ib-cr* and *qnrS2* in the same isolate was observed in 23% ($n = 26$) of the strains (40 isolates had only *aac(6′)-Ib-cr* and 15 isolates had only *qnrS2*). In addition, the variant *aac(6′)-Ib* of the gene *aac(6′)-Ib-cr*, which lacked the two mutations associated with quinolone resistance, was also detected in 13% of the isolates ($n = 15$), mainly in *A. caviae/punctata* ($n = 5$) and *A. hydrophila* ($n = 10$). The genes *aac(6′)-Ib-cr* or *qnrS2* were apparently not associated with a specific taxonomic group, since no significant ($p > 0.05$) difference of gene prevalence was observed. However, isolates recovered on GSP supplemented with 4 mg/L of ciprofloxacin ($n = 74$) presented a significantly ($p < 0.05$) higher frequency of the gene *qnrS2* than isolates from other isolation culture media. The prevalence of isolates harboring the gene *aac(6′)-Ib-cr* was significantly ($p < 0.05$) lower in RWW than in other types of water (Fig. 1, Table S2). Two distinct alleles of this gene, differing in the position 102, were observed, with most of the isolates yielding the codon AGG ($n = 56$) and a minority the codon CGG ($n = 10$). The occurrence of each of those mutations could not be associated with the origin of the isolates.

The gene *qnrS2* was detected in 51% of the wastewater treatment plant isolates ($n = 40$) and was uncommon among hospital effluent isolates, being detected in a single strain identified as *A. hydrophila* (Table S2). Of the bla_{OXA} genes detected, the bla_{OXA-1} was the most common, found in 46% of the isolates ($n = 51$), with no significantly higher prevalence in isolates from any source or taxonomic group, although all the three *A. veronii* isolates had that gene. The gene bla_{OXA-1} was frequently associated with the gene *aac(6′)-Ib-cr* (92% of the isolates with the gene *aac(6′)-Ib-cr*; $n = 47$) or with both *aac(6′)-Ib-cr* and *qnrS2* (43% of the isolates with the gene *aac(6′)-Ib-cr*; $n = 22$) (Table S2). The gene $bla_{OXA-101}$ was detected in seven MDR5 (resistance to at least one antibiotic of five distinct classes of antibiotics) strains identified as *A. caviae* and *A. hydrophila* isolated from hospital effluent ($n = 4$) or treated effluent ($n = 3$), most of which also harbored the genes *aac(6′)-Ib-cr* and/or bla_{OXA-1} (Table S2).

Of the four plasmid replicon types searched for, only incU was detected, in 21% of the isolates ($n = 23$). The presence of incU was associated with the occurrence of the gene *qnrS2* (15% of the isolates, Table S2). Variable regions of class 1 integrons were detected in 39 out of the 112 isolates, most of which ($n = 34$) were shorter than 1000 bp.

Table 1
Prevalence of antibiotic resistance (%) among nalidixic-acid resistant *Aeromonas* spp. isolated from hospital effluent and from the raw inflow and treated effluent of the receiving wastewater treatment plant.

	TIC	MEM	CP	CAZ	GEN	STR	CIP	SXT	TET	CT	MDR ≥ 3	MDR ≥ 4
HE (n = 33)	97.0 a	30.3 a	100.0 a	42.4 a	45.5 a	69.7 a	72.7 a	42.4 a	27.3 a	3.0 a	100.0 a	93.9 a
RWW (n = 43)	76.7 b	16.3 a	86.0 b	7.0 b	20.9 b	39.5 b	62.5 a	16.3 b	11.6 a	0.0 a	88.4 a	67.4 b
TWW (n = 36)	72.2 b	8.3 a	75.0 b	8.3 b	16.7 b	50.0 b	63.9 a	30.6 a	16.7 a	0.0 a	91.7 a	63.9 b
Total (n = 112)	81.3	17.9	86.6	17.9	26.8	51.8	66.1	28.6	17.9	0.9	92.9	74.1

HE, hospital effluent, RWW, raw inflow, TWW, treated effluent. Within a column, values denoted with different letters are significantly different ($p < 0.05$). All isolates were resistant to amoxicillin.

Class 1 integrons variable regions with sizes over 1000 bp contained gene cassettes encoding resistance to streptomycin (*aadA2*) in a MDR5 *A. hydrophila* (HE), to sulfamethoxazole (*dhfr12*) in a MDR5 *A. caviae* (HE), to sulfamethoxazole and beta-lactams (*dfrB4-bla*$_{OXA-10}$) in a MDR4 *A. caviae* (TWW), to aminoglycosides and chloramphenicol (*aadA1-catB8*) in a MDR5 *A. caviae* (TWW), and to aminoglycosides and trimethoprim (*aacA4-dfrA1*) in a MDR5 *A. caviae* (RWW) (Table S2).

3.4. Sources and routes of qnrS2 and aac(6′)-Ib-cr dissemination

Since the gene *aac*(6′)-*Ib-cr* was homogeneously distributed in hospital and municipal wastewater and the gene *qnrS2* was mainly found in municipal effluents, the genotypes of the isolates harboring these genetic determinants were compared, based on a multilocus sequence typing method. In this analysis were included all HE isolates and municipal wastewater isolates harboring both the genes *aac*(6′)-*Ib-cr* and *qnrS2* (n = 54) (Table S2). This analysis suggested that genetically closely related strains can be found in hospital effluent and in municipal wastewater (Fig. 1, Graphical abstract), a fact that is not surprising since the hospital effluent is discharged to this wastewater treatment plant. However, it was observed that genetically closely related strains of the groups *A. hydrophila* (e.g. H1GC4 and E4GC82) and *A. caviae* (e.g. strains H1GC32, H1GA53, E4GC39 and E4GC88) yielded the *qnrS2* gene only when they had origin in the wastewater treatment plant (Fig. 1, Graphical abstract).

Two isolates, out of the nine tested, produced *E. coli* J53-transconjugants with donor strains from HE, with acquired phenotypes of resistance to amoxicillin and cephalothin. One of these transconjugants, in which the donor strain was a HE isolate, harbored the plasmid replicon type IncU and the gene *qnrS2*, associated with an increase in minimum inhibitory concentration (MIC_{CIP}) from 0.015 mg/L to 0.25 mg/L.

4. Discussion and conclusions

Considering that the clinical settings are regarded as important reservoirs of antibiotic resistance, it can be argued that the discharge of hospital effluents into municipal wastewater treatment plants may increase the risks of dissemination of antibiotic resistance (Kümmerer and Henninger, 2003; Varela et al., 2014, 2015a). The environmental dissemination of antibiotic resistance is a complex issue, in which study strategies may involve the comparison of prevalence/abundance values or antibiotic resistance determinants and bacteria over different compartments (Czekalski et al., 2014; Narciso-da-Rocha et al., 2014; Vaz-Moreira et al., 2014). The outcomes of this approach can be valued if relevant environmental antibiotic resistance carriers and genetic determinants are traced over distinct locations. This rationale supports the study of antibiotic resistance in members of the genus *Aeromonas*, with recognized ubiquity in water environments. Indeed, members of this genus, although not belonging to the list of the leading agents of health care associated infections (European Centre For Disease Prevention and Control, 2012), are important antibiotic resistance reservoirs in aquatic environments (Janda and Abbott, 2010; Piotrowska and Popowska, 2014), promising to be good tools to compare hospital and municipal wastewater. In addition, quinolone resistance, which presents a high penetrance in wastewater environments (Figueira et al., 2011; Varela et al., 2015a; Vaz-Moreira et al., 2014), is a promising monitoring tag to assess antibiotic resistance dissemination. Hence, quinolone resistant *Aeromonas* spp. were selected as adequate tools for this study.

Among the PMQR genes screened for, only *aac*(6′)-*Ib-cr* and *qnrS2* were detected, often in combination. However, confirming the initial expectation that quinolone resistant *Aeromonas* spp. could be useful tracers of antibiotic resistance dissemination, different routes of dissemination were demonstrated for those two genes. The gene *qnrS2*, sometimes associated with the plasmid replicon type incU, was frequent in municipal wastewater and rare in hospital effluent, suggesting that its reservoir is not the clinical setting. In contrast, the gene *aac*(6′)-*Ib-cr* was common in both types of water, confirming its ubiquity already suggested in other studies focused on *E. coli* (Varela et al., 2015a; Vredenburg et al., 2014). The association of the gene *aac*(6′)-*Ib-cr* to MDR phenotypes (including resistance to ticarcillin, cephalothin, streptomycin, ciprofloxacin and others such as meropenem or tetracycline) has also been observed before for *E. coli*, which reinforces the idea that this gene is a potential indicator of antibiotic resistance dissemination (Chen et al., 2012; Varela et al., 2015a). *Aeromonas* spp. harboring the gene *aac*(6′)-*Ib-cr* were isolated from treated wastewater, suggesting the good fitness of members of this genus during wastewater treatment and highlighting their potential as vectors of that gene in aquatic environments. The aforementioned evidences suggest that the gene *aac*(6′)-*Ib-cr* in *Aeromonas* spp. may represent an indicator of MDR in wastewater and related environments.

This conclusion contrasts with that observed for the gene *qnrS2*, which was significantly more frequent ($p < 0.05$) in isolates with resistance to antibiotics belonging to less than four distinct classes and had origin mainly in municipal wastewater. This was confirmed by the phylogenetic evidence that demonstrated that municipal wastewater was a reservoir of the gene *qnrS2*. Therefore, the gene *qnrS2* in *Aeromonas* spp. was suggested as an indicator of antibiotic resistance with origin in municipal wastewater.

This conclusion is supported by previous studies demonstrating the occurrence of this genetic element in environmental isolates (Bönemann et al., 2006; Marti and Balcázar, 2012). Although quinolone resistance in frequently associated with mutations in the genes encoding the enzymes DNA gyrase and DNA topoisomerase IV (Strahilevitz et al., 2009), other genetic determinants, predominantly environmental, like *qnrS2* may be important links on the ecology of antibiotic resistance.

Noticeably, in this study, the only isolate from hospital effluent that carried the quinolone resistance gene *qnrS2* showed the ability to transfer it to an *E. coli* receptor. Indeed, genes responsible for low levels of antibiotic resistance, such as *qnrS2* and/or *aac*(6′)-*Ib-cr*, have been suggested to play a role in the maintenance of resistance levels in the population in the presence of sub-inhibitory concentrations of antibiotics (Cantón and Morosini, 2011; Poirel et al., 2012; Rodríguez-Martínez et al., 2011). Therefore, the occurrence of these resistance determinants in environmental isolates deserves attention.

Most isolates with PMQR, in particular with the gene *aac*(6′)-*Ib-cr*, were also found to harbor beta-lactamase genes, mainly *bla*$_{OXA-1}$,

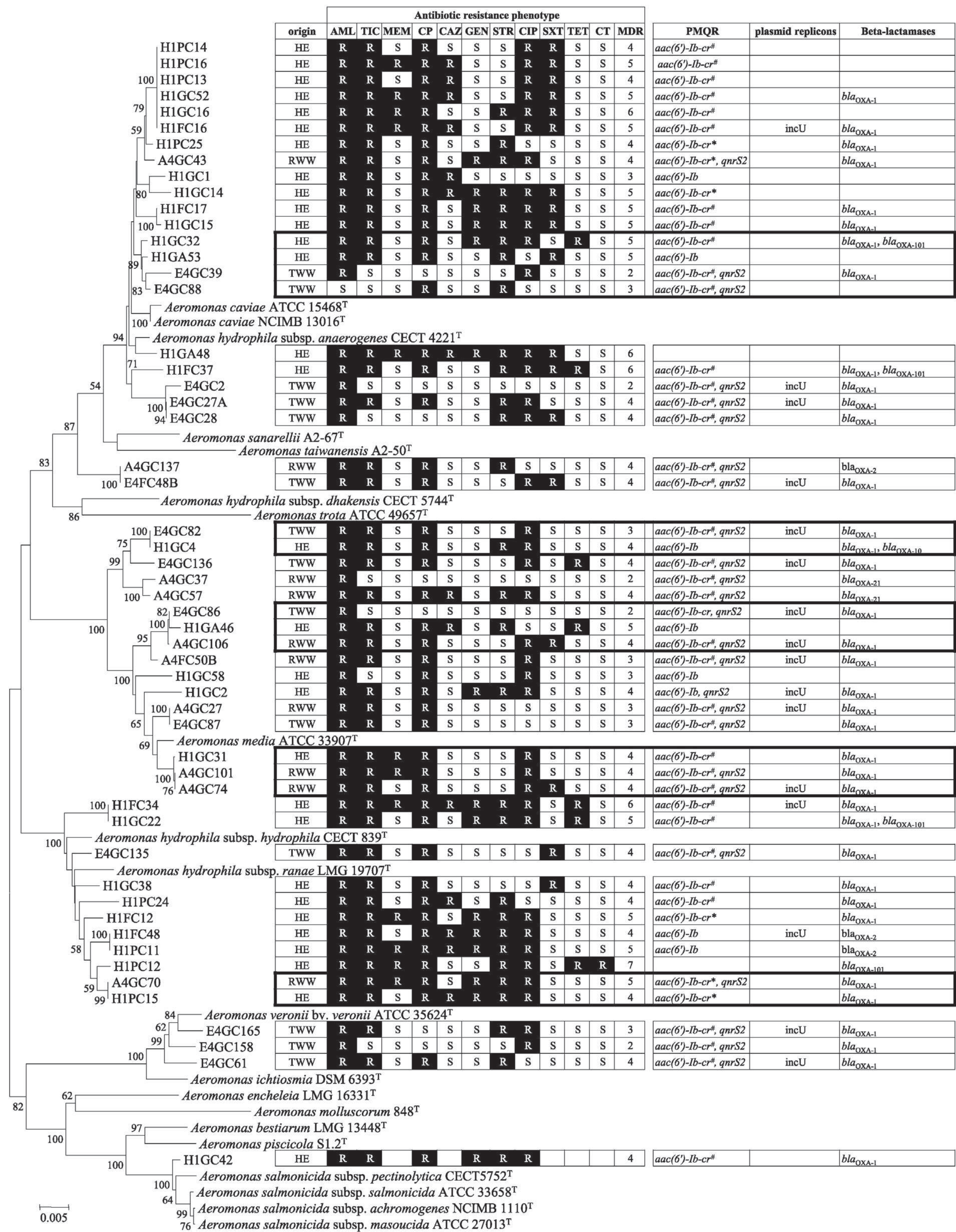

Fig. 1. Neighbor joining dendrogram based on the 16S rRNA, *cpn60* and *gyrB* concatenated nucleotide sequences of 54 nalidixic acid resistant isolates harboring PMQR genes and the closest neighbor type strains available in the GenBank database (accession numbers within brackets) and characteristics of the isolates, regarding origin, antibiotic resistance profile, presence of plasmid mediated quinolone resistance determinants, plasmid replicons and beta-lactamase genes. *mutation TGG > CGG in position 102, #mutation TGG > AGG in position 102. All isolates were resistant to amoxicillin. Isolation conditions (culture media/supplementation with antibiotics) are indicated by the third and fourth letters in isolates name, respectively: G, GSP; P, PCA; F, mFC; A, 32 mg/L amoxicillin; C, 4 mg/L ciprofloxacin. Origin: HE, hospital effluent, RWW, raw inflow and TWW, treated effluent of the receiving wastewater treatment plant; PMQR, plasmid mediated quinolone resistance; TIC, ticarcillin; MEM, meropenem; CP, cephalotin, CAZ, ceftazidime; GEN, gentamycin; STR, streptomycin; CIP, ciprofloxacin; SXT, sulphamethoxazole/trimethoprim; TET, tetracycline; CT, colistin; MDR, multidrug resistance. Outlined, genetically similar isolates carrying different plasmid mediated quinolone resistance determinants.

evidencing an association between both types of determinants that has been reported before (Marti and Balcázar, 2012; Picão et al., 2008). *Aeromonas* spp. are well known carriers of bla_{OXA} genes in food products (Chang et al., 2007), ornamental fish (Dobiasova et al., 2014), clinical samples (Lee et al., 2008) or waterways (Marti and Balcázar, 2012; Tacão et al., 2014). Most of the beta-lactam resistance genes detected in this study, bla_{OXA-1}, bla_{OXA-2}, bla_{OXA-10} and bla_{OXA-21}, have been reported mainly in environmental aquatic isolates (Moura et al., 2012; Picão et al., 2008), confirming the ubiquity of these determinants. Although the sets of primers used for beta-lactamase detection were not designed aiming at the detection of this gene variant (Henriques et al., 2006), it was possible to amplify the gene $bla_{OXA-101}$ of the bla_{OXA-10} family, characterized by possessing two nucleotide changes that result in the substitution of isoleucine for valine in position 89 (GTT → ATT), and of alanine for threonine in position 230 (ACA → GCA) relative to bla_{OXA-56} (Porto et al., 2010). This gene, identified in this work in hospital effluent and wastewater treatment plant isolates, encodes a recently identified beta-lactamase (Porto et al., 2010) that, so far, has only been reported in clinical isolates of *Pseudomonas aeruginosa*, *Citrobacter freundii* and *Enterobacter cloacae* (Juan et al., 2010; Martinez et al., 2014). Therefore, to the best of our knowledge, this is the first report of this gene in *Aeromonas* spp.

This study highlights the importance of *Aeromonas* spp. as tracers of antibiotic resistance in water environments. Members of this genus appear to be highly liable for antibiotic resistance acquisition, being suggested as relevant indicators of antibiotic resistance in the environment. Although hospital and municipal wastewater presented different types of PMQR, the hospital effluent was confirmed as a non-negligible reservoir of antibiotic resistance. The input from hospital effluents puts the native aquatic populations in contact with a whole range of resistant bacteria and genes that can be integrated into the community under favorable conditions such as the presence of a selective pressure constituted by excreted antibiotic residues. Therefore, the impact of hospital wastewater in the dissemination of antibiotic resistance in the environment should be carefully considered.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.10.124.

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Appendix A – Supplementary information

Manuscript title: Quinolone resistant *Aeromonas* spp. as carriers and potential tracers of acquired antibiotic resistance in hospital and municipal wastewater

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Table S2 – Origin, antibiotic resistance profile, presence of antibiotic resistance genes, plasmid replicons and class I integrons for the isolates included in the study.

Table S1 - Groups of species comprising the isolates included in this study according to the identification retrieved in the EzTaxon database and with which presented 16S rRNA gene sequence similarity values ≥ 99%.

Taxonomic group	Closest neighbour (type strain)
Aeromonas caviae/punctata	*Aeromonas punctata* subsp. *punctata* (NCIMB 13016^{T}) *Aeromonas punctata* subsp. *caviae* (ATCC 15468^{T}) *Aeromonas caviae* (CECT 4221^{T}) *Aeromonas enteropelogenes/trota* (ATCC 49657^{T}) *Aeromonas dhakensis* (LMG 19562^{T}) *Aeromonas sanarellii* (A2-67^{T}) *Aeromonas taiwanensis* (A2-50^{T})
Aeromonas veronii	*Aeromonas veronii* (ATCC 35624^{T}) *Aeromonas ichthiosmia* (DSM 6393^{T})
Aeromonas salmonicida	*Aeromonas salmonicida* subsp. *salmonicida* (ATCC 33658^{T}) *Aeromonas salmonicida* subsp. *masoucida* (ATCC 27013^{T}) *Aeromonas salmonicida* subsp. *achromogenes* (NCIMB 1110^{T}) *Aeromonas bestiarum* (CIP 7430^{T}) *Aeromonas piscicola* (S1.2^{T}) *Aeromonas salmonicida* subsp. *pectinolytica* (34mel^{T}) *Aeromonas encheleia (*LMG 16331^{T}) *Aeromonas molluscorum* (848^{T})
Aeromonas hydrophila	*Aeromonas hydrophila* subsp.*hydrophila* (ATCC 7966^{T}) *Aeromonas hydrophila* subsp. *ranae* (LMG 19707^{T}) *Aeromonas media* (ATCC 33907^{T})
Aeromonas allosacharophila	*Aeromonas allosaccharophila* (CECT 4199^{T})

Table S2 – Origin, antibiotic resistance profile, presence of antibiotic resistance genes, plasmid replicon type and class I integrons for the isolates included in the study.

Isolate	origin	Antibiotic resistance profile	PMQR					Plasmid replicons	Beta-lactamases	Class 1 integrons
			aac(6')-Ib				*qnr*			
			Variant	pos102	pos117	pos179				
A4FC4		TIC (2)								
A4FC27		TIC (2)								
A4FC32		TIC, CP, STR, CIP, SXT (5)	*aac(6')-Ib*	W(TGG)	S(TCA)	D (GAT)			*bla*$_{OXA-1}$, *bla*$_{OXA-10}$	*aacA4-dfrA1-orf*
A4FC50B		TIC, CP, CIP (3)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*	incU	*bla*$_{OXA-1}$	~200bp
A4FC59B		CP, STR (4)								
A4FC89B		CP, STR, SXT, TET (6)								
A4FC95		TIC, CP, GEN, CIP (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)		incU		~500 bp
A4GA12		(2)								
A4GA15		CP, SXT, TET (5)								
A4GA16y		TIC, CP (3)	*aac(6')-Ib*	W(TGG)	S(TCA)	D (GAT)				
A4GA29		TIC, MEM, CP, STR, CIP (5)					*qnrS2*			
A4GA42		TIC, CP, GEN (4)								
A4GA49		TIC, CP (3)								
A4GA53x		TIC, MEM, CP (4)								
A4GC13x		TIC, MEM, CP, SXT, TET (6)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			*bla*$_{OXA-1}$, *bla*$_{OXA-10}$	
A4GC17	RWW	TIC, CP, STR, CIP (4)					*qnrS2*			
A4GC18		CP, STR, CIP (4)					*qnrS2*			
A4GC24		CP, STR, CIP (4)								
A4GC25		TIC, CP, GEN, CIP (4)	*aac(6')-Ib*	W(TGG)	L(TTA)	Y(TAT)			*bla*$_{OXA-10}$	
A4GC27		TIC, CP (3)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*	incU	*bla*$_{OXA-1}$	~200bp
A4GC37		(2)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*		*bla*$_{OXA-21}$	~200bp
A4GC39		CP (3)					*qnrS2*		*bla*$_{OXA-2}$	
A4GC43		TIC, CP, GEN, STR, CIP (4)	*aac(6')-Ib-cr*	R(CGG)	L(TTA)	Y(TAT)	*qnrS2*		*bla*$_{OXA-1}$	~200bp
A4GC57		TIC, CP, CAZ, STR, CIP (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*		*bla*$_{OXA-21}$	
A4GC58		TIC, CP, CIP (3)					*qnrS2*			
A4GC59		CIP (2)					*qnrS2*	incU	*bla*$_{OXA-1}$	~200bp
A4GC60		TIC, CP, GEN, STR, CIP, TET (5)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			*bla*$_{OXA-1}$	
A4GC61		TIC, CP, STR, CIP (4)	*aac(6')-Ib*	W(TGG)	L(TTA)	Y(TAT)	*qnrS2*			
A4GC62		TIC, CP, GEN, STR, CIP (4)								
A4GC69		TIC, MEM, CP, STR, CIP (5)								
A4GC70		TIC, MEM, CP, GEN, STR, CIP (5)	*aac(6')-Ib-cr*	R(CGG)	L(TTA)	Y(TAT)	*qnrS2*		*bla*$_{OXA-1}$	~200bp
A4GC74		TIC, CP, CIP, SXT (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*	incU	*bla*$_{OXA-1}$	

A4GC76		TIC, CP, CIP (3)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*	incU	*bla*$_{\text{OXA-1}}$	~200bp
A4GC86		TIC, CP, CAZ, SXT (4)					*qnrS2*			
A4GC87		TIC, MEM, CP, CIP (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*		*bla*$_{\text{OXA-1}}$	~200bp
A4GC99		TIC, CP, CIP (3)					*qnrS2*			
A4GC101		TIC, MEM, CP, CIP (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*		*bla*$_{\text{OXA-1}}$	
A4GC106		TIC, CP, CIP, SXT (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*	incU	*bla*$_{\text{OXA-1}}$	~200bp
A4GC118		TIC, CP, GEN, STR, CIP (4)	*aac(6')-Ib-cr*	R(CGG)	L(TTA)	Y(TAT)			*bla*$_{\text{OXA-1}}$	~200bp
A4GC120		TIC, CP, CIP, TET (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)				
A4GC131		CAZ, CIP (3)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)				
A4GC137		TIC, CP, STR (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*		*bla*$_{\text{OXA-2}}$	
A4GC138		TIC, CP, GEN, STR, CIP (4)	*aac(6')-Ib-cr*	R(CGG)	L(TTA)	Y(TAT)			*bla*$_{\text{OXA-1}}$	~200bp
E4FC6		TIC, CP, GEN, STR, CIP (4)	*aac(6')-Ib*	W(TGG)	L(TTA)	D (GAT)				
E4FC11		TIC, CP, CAZ, GEN, STR, CIP, SXT (5)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			*bla*$_{\text{OXA-1}}$, *bla*$_{\text{OXA-101}}$	*aadA1-catB8*
E4FC44		TIC, MEM, CP, CAZ, STR, CIP, SXT, TET (7)	*aac(6')-Ib-cr*	R(AGG)	S(TCA)	D (GAT)				~900bp
E4FC48B		TIC, CP, CIP, SXT (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*	incU	*bla*$_{\text{OXA-1}}$	
E4FC67		TIC, CP, STR, CIP (4)	*aac(6')-Ib-cr*	R(CGG)	L(TTA)	Y(TAT)			*bla*$_{\text{OXA-1}}$	~200bp
E4GC2		(2)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*	incU	*bla*$_{\text{OXA-1}}$	
E4GC3		TIC, CP, CIP (3)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)		incU		
E4GC8		CP, TET (4)								
E4GC9		STR, TET (4)								
E4GC10		TIC, CP, STR, CIP, SXT (5)								
E4GC17		TIC, CP, CIP (3)								
E4GC18		CP (3)					*qnrS2*	incU		~700bp
E4GC21		CP, STR (4)					*qnrS2*		*bla*$_{\text{OXA-2}}$	
E4GC27A	TWW	TIC, CP, STR, CIP (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*	incU	*bla*$_{\text{OXA-1}}$	
E4GC28		STR, CIP, SXT (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*	incU	*bla*$_{\text{OXA-1}}$	
E4GC37		SXT (3)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			*bla*$_{\text{OXA-1}}$	
E4GC39		CIP (2)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*		*bla*$_{\text{OXA-1}}$	~200bp
E4GC41		TIC, GEN, STR, CIP, TET (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			*bla*$_{\text{OXA-101}}$	
E4GC42		TIC, CP, GEN, STR, CIP (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)				
E4GC51		TIC, CP, CIP (3)					*qnrS2*			
E4GC61		TIC, CP, STR (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*	incU	*bla*$_{\text{OXA-1}}$	~200bp
E4GC80		TIC, CP, CIP (3)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)				
E4GC81		TIC, MEM, CP, SXT (5)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)		incU	*bla*$_{\text{OXA-1}}$	
E4GC82		TIC, CP, CIP (3)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*	incU	*bla*$_{\text{OXA-1}}$	~200bp
E4GC86		(2)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*	incU	*bla*$_{\text{OXA-1}}$	~200bp
E4GC87		TIC, CP (3)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*		*bla*$_{\text{OXA-1}}$	~200bp
E4GC88		CP, STR, SXT (5)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*			
E4GC97		TIC, CP, CIP, SXT (4)					*qnrS2*		*bla*$_{\text{OXA-10}}$	*dfrB4-bla*$_{\text{OXA-10}}$

E4GC130		TIC, CP, STR, TET (5)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			bla_{OXA-1}, $bla_{OXA-101}$	~500 bp
E4GC135		TIC, CP, SXT (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*		bla_{OXA-1}	
E4GC136		TIC, CP, CIP, TET (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*	incU	bla_{OXA-1}	~200bp
E4GC148		TIC, CP, GEN, STR, CIP, SXT (5)	*aac(6')-Ib*	W(TGG)	S(TCA)	Y(TAT)				
E4GC150		TIC, CP, CAZ, GEN, CIP (4)	*aac(6')-Ib*	W(TGG)	S(TCA)	D (GAT)			bla_{OXA-1}	~200bp
E4GC158		TIC, MEM, CP, STR, CIP (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*		bla_{OXA-1}	~200bp
E4GC165		TIC, STR, CIP (3)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*qnrS2*	incU	bla_{OXA-1}	~200bp
E4GC166		TIC, STR, CIP (3)					*qnrS2*			
H1FC12		TIC, MEM, CP, GEN, STR, CIP (5)	*aac(6')-Ib-cr*	R(CGG)	L(TTA)	Y(TAT)			bla_{OXA-1}	
H1FC16		TIC, MEM, CP, CAZ, CIP, SXT (5)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)		incU	bla_{OXA-1}	
H1FC17		TIC, CP, GEN, STR, CIP, SXT (5)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			bla_{OXA-1}	*dhfr12-orf*
H1FC32		TIC, CP, CAZ, GEN, STR, TET (5)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			bla_{OXA-1}	
H1FC34		TIC, MEM, CP, CAZ, GEN, STR, CIP, TET (6)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)		incU	bla_{OXA-1}	~900bp
H1FC37		TIC, CP, STR, CIP, SXT, TET (6)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			bla_{OXA-1}, $bla_{OXA-101}$	
H1FC48		TIC, CP, CAZ, GEN, STR, CIP (4)	*aac(6')-Ib*	W(TGG)	L(TTA)	D (GAT)		incU	bla_{OXA-2}	~900bp
H1GA23		TIC, CP, TET (4)	*aac(6')-Ib-cr*	R(CGG)	L(TTA)	Y(TAT)				
H1GA46		TIC, CP, CAZ, STR, TET (5)	*aac(6')-Ib*	W(TGG)	S(TCA)	D (GAT)				~900bp
H1GA48		TIC, MEM, CP, CAZ, GEN, STR, CIP, SXT (6)								
H1GA53		TIC, CP, STR, SXT (5)	*aac(6')-Ib*	W(TGG)	S(TCA)	D (GAT)				
H1GC1		TIC, CP, CAZ (3)	*aac(6')-Ib*	W(TGG)	S(TCA)	D (GAT)				
H1GC2		TIC, CP, GEN, STR, CIP (4)	*aac(6')-Ib*	W(TGG)	S(TCA)	D (GAT)	*qnrS2*	incU	bla_{OXA-1}	~500 bp
H1GC4		TIC, CP, STR, CIP (4)	*aac(6')-Ib*	W(TGG)	S(TCA)	D (GAT)			bla_{OXA-10}	
H1GC5		TIC, CP, GEN, STR, SXT, TET (6)	*aac(6')-Ib*	W(TGG)	L(TTA)	Y(TAT)				
H1GC14	HE	TIC, CP, CAZ, GEN, STR, CIP, SXT (5)	*aac(6')-Ib-cr*	R(CGG)	L(TTA)	Y(TAT)				
H1GC15		TIC, CP, GEN, STR, CIP, SXT (5)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			bla_{OXA-1}	~900 bp
H1GC16		TIC, MEM, CP, STR, CIP, SXT (6)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)				
H1GC22		TIC, CP, GEN, STR, CIP, TET (5)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			bla_{OXA-1}, $bla_{OXA-101}$	*aadA2*
H1GC31		TIC, MEM, CP, CIP (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			bla_{OXA-1}	
H1GC32		TIC, CP, GEN, STR, CIP, TET (5)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			bla_{OXA-1}, $bla_{OXA-101}$	
H1GC38		TIC, CP, SXT (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			bla_{OXA-1}	
H1GC42		TIC, CP, GEN, STR, CIP (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			bla_{OXA-1}	
H1GC52		TIC, MEM, CP, CAZ, CIP, SXT (5)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)			bla_{OXA-1}	
H1GC58		CP, CIP (3)	*aac(6')-Ib*	W(TGG)	S(TCA)	D (GAT)				~900 bp
H1PC11		TIC, MEM, CP, CAZ, GEN, STR, CIP (5)	*aac(6')-Ib*	W(TGG)	S(TCA)	D (GAT)			bla_{OXA-2}	~900 bp
H1PC12		TIC, MEM, CP, STR, CIP, TET, CT (7)							$bla_{OXA-101}$	
H1PC13		TIC, CP, CAZ, CIP, SXT (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)				
H1PC14		TIC, CP, CIP, SXT (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)				
H1PC15		TIC, CP, CAZ, GEN, STR, CIP (4)	*aac(6')-Ib-cr*	R(CGG)	L(TTA)	Y(TAT)			bla_{OXA-1}	
H1PC16		TIC, MEM, CP, CAZ, CIP, SXT (5)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)				

H1PC24	TIC, CP, CAZ, STR (4)	*aac(6')-Ib-cr*	R(AGG)	L(TTA)	Y(TAT)	*bla*$_{OXA-1}$	~500 bp
H1PC25	TIC, CP, STR (4)	*aac(6')-Ib-cr*	R(CGG)	L(TTA)	Y(TAT)	*bla*$_{OXA-1}$	~500 bp

Caption: All isolates were resistant to amoxicillin and nalidixic acid. Isolation conditions (culture media/supplementation with antibiotics) are indicated by the third and fourth letters in isolates name, respectively: G, GSP; P, PCA; F, mFC; A, 32 mg/L amoxicillin; C, 4 mg/L ciprofloxacin. Origin: HE, hospital effluent, RWW and TWW, raw inflow and treated effluent of the receiving wastewater treatment plant; TIC, ticarcillin; MEM, meropenem; CP, cephalotin, CAZ, ceftazidime; GEN, gentamycin; STR, streptomycin; CIP, ciprofloxacin; SXT, sulphamethoxazole/trimethoprim; TET, tetracycline; CT, colistin; MDR, resistance to different classes of antibiotics. PMQR – plasmid mediated quinolone resistance; R, arginine; W, tryptophan; S, serine; L, leucine; D, aspartic acid; Y, tyrosine. Shadowed cells correspond to the isolates presented in Figure 1.

Chapter 5

Influence of sub-inhibitory concentrations of antibiotics in the transfer rate of the conjugative plasmidome of a hospital effluent multidrug resistant isolate

(submitted for publication)

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Abstract

This study aimed at analyzing the effect of sub-inhibitory concentrations of ceftazidime or/and tetracycline in the conjugation efficiency of an *Escherichia coli* isolate originally recovered from hospital effluent, strain H1FC54. In addition, it was aimed at evaluating the whole set of resistance traits that can be putatively acquired through conjugation, therefore, allowing inferences on the co-selection potential of antibiotics. With these aims, the donor strain H1FC54 was mated with the azide resistant *E. coli* J53, and the rate of conjugation in the presence and absence of antibiotics and the genetic composition of the conjugative plasmidome were determined.

Transconjugants acquired phenotypes of resistance to amoxicillin, ticarcillin, cephalothin, sulfamethoxazole and tetracycline, an increased minimal inhibitory concentration (MIC) of ciprofloxacin and ceftazidime, and genetic determinants of plasmid replicons and resistance to antibiotics. The presence of sub-inhibitory concentrations of ceftazidime or tetracycline were associated, respectively, with significantly ($\alpha<0.05$) higher or lower conjugation rates than those observed in the presence of other conditions.

The analysis of the nucleotide sequence of the conjugative plasmidome of strain H1FC54 revealed the presence of a HI2 plasmid backbone with determinants of resistance to a wide range of antibiotics (aminoglycosides, beta-lactams, (fluoro)quinolones, tetracyclines, chloramphenicol, sulfonamides, rifampicin), to ethidium bromide, tellurite, mercury and arsenic compounds, copper, nickel and cobalt, and a cobalt-zinc-cadmium resistance protein, as well as genes associated with bacterial persistence. The observation of such an array of putative functions related with resistance and persistence in the environment, combined in a single conjugative plasmidome, whose transfer can be enhanced in the presence of sub-inhibitory concentrations of ceftazidime, is an important evidence of the potential of dissemination of resistance genes from wastewater to other environmental bacteria.

Introduction

By definition, acquired antibiotic resistance refers to genes located in mobile genetic elements and which, therefore, can be mobilized between the mobile genetic element and the bacterial chromosome or between different bacterial cells (Normark & Normark 2002). The mobile resistome includes phages, naked DNA and, the most studied, a wide diversity of plasmids (Manaia et al. 2012). Resistance genes carried by plasmids confer resistance to most classes of antibiotics, including beta-lactams, quinolones, macrolides, tetracyclines, sulfonamides among others (Szczepanowski et al. 2009). The risk for human health associated with plasmid mobility relies on their potential for spreading traits such as antibiotic resistance and/or virulence phenotypes, often acquired by pathogenic bacteria through conjugative plasmids (Giakkoupi et al. 2015).

It has been argued that the occurrence of antibiotics as environmental contaminants from human origin may contribute in several ways to the emergence and dissemination of antibiotic resistance, for example by causing the selection of antibiotic resistant bacteria (Andersson & Hughes 2010). In particular, it has been suggested that exposition of bacteria to sub-inhibitory concentrations of antibiotics may stimulate horizontal gene transfer and consequently lead to the increase of the dissemination of antibiotic resistance genes (Andersson & Hughes 2014). Since sub-inhibitory concentrations of antibiotics, as well as bacteria harboring conjugative plasmids encoding antibiotic resistance, occur frequently in wastewater, the underlying hypothesis of this study was if such conditions are implied in resistance dissemination through conjugation. To test this hypothesis, a multidrug resistant isolate from hospital effluent that in previous studies showed the capacity to transfer resistance traits to a known recipient (Varela et al. 2015), was used as a model for conjugation assays conducted in the presence of sub-inhibitory concentrations of ceftazidime or/and tetracycline. The model strain, *E. coli* H1FC54, displayed resistance to antibiotics from six different classes, and tested positive for the presence of genes associated with resistance to quinolones and beta-lactams, and plasmid replicons from the groups HI2, FIA and FIB (Varela et al., 2015). Hence, this strain and its plasmidome seemed an interesting model to assess the potential for transmission of antibiotic resistance genes in the presence of sub-inhibitory concentrations of antibiotics, as those that can be found in wastewater compartments. In addition, it was aimed to explore the genetic content of the transferred plasmidome as a way of assessing the set of traits that can be transferred

and whose transfer can be enhanced in the presence of the selective pressures exerted by antibiotics.

Materials and methods

Bacterial strains, conjugation assays and plasmid isolation

The multidrug resistant *E. coli* strain H1FC54, isolated from hospital effluent, belonging to the multi-locus sequence type ST 93 and harboring quinolone and beta-lactam resistance genes, and replicon type plasmids FIA, FIB and HI2, was used as donor strain in this study (Varela et al. 2015). The recipient strain was the azide resistant *E. coli* J53. Cultures of donor and recipient strains were grown in Luria-Bertani (LB) broth for 4 hours, until the late-exponential growth phase, corresponding to an optical density (OD_{600}) between 0.6-1.0 (Sezonov et al. 2007). These conditions were selected in order to maximize conjugation frequencies (Muela et al. 1994). For mating assays, donor and recipient cultures were mixed in a proportion 1:1 of optical density units, centrifuged at 10000 rpm for 5 min, suspended in fresh LB medium or in this medium supplemented with ceftazidime (10 mg/L) or/and tetracycline (8 mg/L) and incubated for 20 h at 28 ºC. The concentration of antibiotics used was the maximal non-inhibitory concentration for the recipient strain *E. coli* J53. To enumerate the number of viable donor cells in each mating assay before incubation, decimal dilutions (1 to 8) of the mixed suspension were plated on LB agar supplemented with ceftazidime (16 mg/L) and tetracycline (16 mg/L) and incubated for 24 h. After the incubation period, putative transconjugants were selected on LB agar medium supplemented with sodium azide (100 mg/L), ceftazidime (16 mg/L) and tetracycline (16 mg/L), after incubation at 37 ºC for 24 hours. In order to confirm if the recovered colonies were effectively transconjugants, the clones were characterized based on random amplified polymorphic DNA genotyping as described in Ferreira da Silva et al. (2006). In addition, transconjugants were characterized for their antibiotic resistance phenotypes, based on the disk diffusion method or using strips embedded with an antibiotic gradient (MIC Evaluator, Oxoid) and for the presence of plasmid replicon types and antibiotic resistance determinants known to exist in the donor strain, as described previously (Varela et al. 2015). The frequency of conjugation was expressed as the percentage of times conjugation was successful per the total number of conjugation assays and the rate of conjugation was expressed as the ratio of number of transconjugant versus donor colonies.

Plasmidome DNA extraction and analysis

Plasmid DNA was extracted from a transconjugant clone obtained in the presence of 10 mg/L ceftazidime (herein named as h1fc54.tc.caz2, Table 1), using the GRS Plasmid Purification Kit - Maxi (GRISP, Porto, Portugal), according to the manufacturer's instructions. The plasmid DNA extract was further analyzed based on Ion Torrent sequencing (Personal Genome Machine, PGM, Life Technologies, Thermo Fisher, Waltham, MA). Nucleotide sequencing generated 212 196 high quality (Phred score>=20) reads with the average size of 280 bp that were submitted to the ARC (Assembly by Reduced Complexity) (Hunter et al. 2015) pipeline, an iterative, reference guided *de novo assembly* using bowtie2 (Langmead & Salzberg 2012) and SPADES (Bankevich et al. 2012). Annotation was performed using of the Rapid Annotation Subsystem Technology (RAST) (Aziz et al. 2008) server for gene predictions, followed by manual curation based on Basic Local Alignment Search Tool (BLAST) (Johnson et al. 2008) query. Nucleotide sequences screened for antibiotic resistance genes and integrons were crossed-checked with the output obtained by query of the ResFinder (Zankari et al. 2012), VirulenceFinder (Joensen et al. 2014) and INTEGRALL (Moura et al. 2009) databases

Statistical analysis

Data on conjugation efficiency in the presence or absence of different sub-inhibitory concentrations of tetracycline and/or ceftazidime was analyzed using the non-parametric Kruskal-Wallis multiple comparisons ($\alpha<0.05$). Calculations were performed using SPSS 22.0 for Windows (SPSS Inc., Chicago, IL).

Results

Conjugation experiments

The strain *E. coli* H1FC54 presented a frequency of conjugation, *i.e.* number of successful plasmidome transfers in the total of mating assays, above 90% (116 out of the 126 assays). The ten unsuccessful conjugation assays were observed in the absence of antibiotics (n=1), in the presence of 10 mg/L ceftazidime (n=4), in the presence of 8 mg/L tetracycline (n=3) or in the presence of both antibiotics (n=2). The number of transconjugant colonies recovered on the selection medium varied from 2.1 x10^2 CFU/mL

to 4.1 x 10^3 CFU/mL. The presence of antibiotics in the conjugation medium was observed to be related with the variations in the conjugation efficiency, which was found to be significantly higher ($\alpha<0.05$) in the presence of 10 mg/L ceftazidime and significantly lower ($\alpha<0.05$) in the presence of in 8 mg/L tetracycline than in other conjugation conditions; but not significantly different when mating was performed in the absence of antibiotics or in presence of both antibiotics (Table 2). The antibiotic resistance phenotypes of the transconjugants revealed that resistance to the beta-lactams amoxicillin, ticarcillin and cephalotin, to sulfamethoxazole and to tetracycline was transferred in all successful mating events (Table 1). The quinolone resistance gene *aac(6')-Ib-cr* and the beta-lactam resistance genes *bla*$_{OXA-1}$ and *bla*$_{SHV-12}$, as well as the plasmid replicon HI2, were transferred in all successful mating events (Table 1). In addition, the amplicons corresponding to the plasmid replicons FIA and FIB and to the beta-lactam resistance gene *bla*$_{TEM}$ were detected occasionally in transconjugants obtained from mating experiences in the absence of antibiotics (Table 1). The acquisition of genetic elements of resistance through conjugation coincided with an increase of the minimum inhibitory concentration of ciprofloxacin (MIC_{CIP}) and of ceftazidime (MIC_{CAZ}) of the transconjugants, in comparison to the recipient strain *E.coli* J53 (MIC_{CIP} 0.015 μg/mL, MIC_{CAZ} 0.064 μg/mL) to values ranging 0.12-0.25 μg/mL and 3.5-8 μg/mL, respectively (Table 2).

Closest relatives of the analyzed plasmidome

In order to get additional information about the conjugative plasmidome of strain *E. coli* H1FC54, the nucleotide sequence of plasmid DNA extracted from a transconjugant (h1fc54.tc.caz2) originated in the presence of 10 mg/L ceftazidime was analyzed (Table 3). It was possible to assemble 143 514 reads into 23 contigs, in a total of 422 724 nucleotides. Further analyses were performed based on this preliminary assemblage. The antibiotic resistance genes and plasmid replicon type regions previously identified based on targeted PCR reactions in the donor strain and in the transconjugants (Table 1) were also detected in the plasmidome nucleotide sequences. Based on the comparison of this plasmidome DNA with other plasmids available in public databases, it was possible to find regions with high sequence similarity (above 97 %) with plasmid encoded genes associated with functions such as plasmid replication, partitioning and transfer, antibiotic resistance or plasmid persistence. The detection in the plasmidome nucleotide sequences of replicon types HI2 and HIA, which is indicative of a HI-type plasmid (Page et al., 2001), was a

confirmation of the previous PCR-based detection of the HI2 type plasmid in strain H1FC54. For the regions associated with conjugation, transfer and replication, the conjugative plasmidome of strain *E. coli* H1FC54 showed highest sequence similarity with the plasmid pR478 (BX664015) of the incompatibility group H, originally described in a clinical isolate of *Serratia marcescens* (Medeiros & O'Brien 1969; Gilmour et al. 2004). The plasmidome analyzed in the current study and pR478 (BX664015) differed on the structure of the region corresponding to the transfer region I of pR478 (BX664015), which in the H1FC54 conjugative plasmidome was separated in two segments, detected in two different non-overlapping contigs (named as transfer regions I and II in Table 3) (Fig.1). The transfer region II, whose homologue in pR478 (BX664015) is adjacent to the homologue of transfer region I, was flanked on the left by an IS*903*-like transposase (97.8% nucleotide sequence similarity), which suggests the separation of transfer regions I and II in the H1FC54 conjugative plasmidome may be the result of a transposition event. Other differences of the transfer region of the H1FC54 conjugative plasmidome in comparison to pR478 (BX664015) included the sequences of the genes *parM* and *trhL*, which in H1FC54 were disrupted by the insertion of transposases; and the absence of a repeat inside the repeat region *parS* in H1FC54 (Fig.1).

Other plasmids available in public databases yielded regions with high nucleotide sequence similarity (>97%) with the transfer and conjugation regions of studied plasmidome, for example the HI2 plasmids CP008899, CP008906, EU855787, EU855788; the FIB plasmids KP75077; or untyped plasmids (CP012170) from a clinical isolates of *Enterobacter cloacae*; the HI2 plasmids from *Klebsiella pneumoniae* (EF382672) and from *Salmonella enterica* subsp. *enterica* sv. Typhimurium (KT334335, LN794248, LK056646) of clinical origin; the HI2 plasmids from *Salmonella enterica* subsp. *enterica* sv. Typhimurium (KM877269), sv. Heidelberg (JN983042) and sv. Infantis (LN555650) and from *E. coli* (KT347600) from food production animals; and one untyped plasmid from *Salmonella enterica* subsp. *enterica* sv. Cubana (CP006056) from food (fresh alfafa sprouts).

Table 1 – Characteristics of the strain *Escherichia coli* H1FC54, of the recipient strain *E. coli* J53 and respective transconjugants.

		RAPD profile	Antibiotic resistance profile										Plasmid replicons				Resistance genes			
			AML	TIC	CP	STR	SUL	SXT	TET	MDR	MIC_{CIP} (µg/mL)	MIC_{CAZ} (µg/mL)	FIA	FIB	FII	HI2	PMQR	beta-lactamase		
H1FC54 (donor)			R	R	R	R	R	R	R	6	>32	16	FIA	FIB	FII	HI2	*aac(6')-Ib-cr*	bla_{OXA-1}	bla_{TEM}	bla_{SHV-12}
J53 (recipient)			S	S	S	S	S	S	S	0	0.015	0.064								
transconjugants	h1fc54.tc3	J53	R	R	R	S	R	S	R	4	0.12	6	FIA	FIB		HI2	*aac(6')-Ib-cr*	bla_{OXA-1}	bla_{TEM}	bla_{SHV-12}
	h1fc54.tc5	J53	R	R	R	S	R	S	R	4	0.25	8				HI2	*aac(6')-Ib-cr*	bla_{OXA-1}		bla_{SHV-12}
	h1fc54.tc.caz2	J53	R	R	R	S	R	S	R	4	0.25	3.5				HI2	*aac(6')-Ib-cr*	bla_{OXA-1}		bla_{SHV-12}

RAPD, random amplified polymorphic DNA typing; tc, transconjugants obtained in the absence of antibiotic; tc.caz, transconjugants obtained in the presence of ceftazidime (10 mg/L) and tetracycline (8 mg/L); all isolates were susceptible to meropenem, gentamicin and colistin. AML, amoxicillin; TIC, ticarcillin; CP, cephalothin, CAZ, ceftazidime; STR, streptomycin; SXT, sulfamethoxazole/trimethoprim; SUL, sulfamethoxazole; TET, tetracycline. MDR, number of classes of antibiotics (penicillin, cephalosporin, aminoglycosides sulfonamide, fluoroquinolones or tetracyclines) to which resistance was detected; MIC, minimum inhibitory concentration; PMQR, plasmid-encoded quinolone resistance.

Table 2 – Efficiency of conjugation between the donor strain *Escherichia coli* H1FC54 and the recipient strain *E. coli* J53 in Luria-Bertani broth and in this medium supplemented with antibiotic, at 28 ºC during 20 hours.

Conjugation conditions	Conjugation frequency (%)		Conjugation rate (transconjugants/donors)	
			Mean±SD	Range
Antibiotic-free	97	(35/36)	$2.7 \times 10^{-6\,c} \pm 5.83 \times 10^{-6}$	2.0×10^{-8} - 2.3×10^{-5}
Ceftazidime (10 mg/L)	89	(32/36)	$1.2 \times 10^{-5\,a} \pm 1.60 \times 10^{-5}$	5.2×10^{-8} - 5.5×10^{-5}
Tetracycline (8 mg/L)	89	(24/27)	$2.5 \times 10^{-7\,b} \pm 2.30 \times 10^{-7}$	2.8×10^{-8} - 7.5×10^{-7}
Ceftazidime (10 mg/L) + Tetracycline (8 mg/L)	93	(25/27)	$7.6 \times 10^{-7\,c} \pm 8.51 \times 10^{-7}$	3.7×10^{-8} - 2.7×10^{-6}
Total	92	(116/126)	$4.3 \times 10^{-6} \pm 1.01 \times 10^{-5}$	2.0×10^{-8} - 5.5×10^{-5}

[a]significantly ($\alpha < 0.05$) higher or [b]lower than conjugation efficiency under other conditions

Genetic determinants of antibiotic and metal resistance

The plasmidome analyzed in this study had regions with high similarity with putative resistance determinants to aminoglycosides, beta-lactams, (fluoro)quinolones, tetracyclines, chloramphenicol, sulfonamides, rifampicin, ethidium bromide, tellurite, mercury and arsenic compounds, copper, nickel and cobalt, and a cobalt-zinc-cadmium resistance protein (Table 3). In addition to conjugation, transfer and replication regions, resistance encoding regions of the H1FC54 conjugative plasmidome with high sequence similarity with regions of the plasmid pR478 (BX664015) were also present. These regions included the clusters of genes associated with resistance to tellurite (*terY3Y2XY1W-[6 orf]-terZABCDEF*, >99% nucleotide sequence similarity), to mercury (*merEDACPTR*, >99.7% nucleotide sequence similarity), and to arsenic compounds and sulfate (*arsHRBC-sfpBA*, associated with a transposase, 100% nucleotide sequence similarity) (Table 3). The cluster of genes associated with tellurite resistance is a common feature of HI2 plasmids (Gilmour et al. 2004; Nies 1999) and is characterized by a conserved set of sequences (*terY3Y2XY1W-[6 orf]-ZABCDEF*, with six conserved genes of unknown function in the region between *terW* and *terZ*). In addition, the analyzed plasmidome contained copper resistance-like determinants (*pcoE, pcoS*) that yielded 90-98% nucleotide sequence similarity with the corresponding regions in pR478 (BX664015), linked to genes encoding resistance to nickel and cobalt (*rcnA- rcnR*), not detected in pR478 (BX664015) (Table 3). Although pR478 (BX664015) carried genetic determinants of resistance to tetracycline (*tetD, tetA, tetR*) and a IS*26*-associated chloramphenicol resistance gene (*cat*), these elements shared less than 60% nucleotide sequence similarity with the corresponding genetic determinants ((*tetA(D), catA2*) in the conjugative plasmidome of strain H1FC54, suggesting a different origin for these genes.

Most of the resistance determinants found in the conjugative plasmidome of strain H1FC54 were concentrated in a multidrug resistance region spanning 17 110 bp. This region included genes conferring resistance to aminoglycosides, (fluoro)quinolones, beta-lactams, chloramphenicol, rifampicin, ethidium bromide, sulfonamides and a mercury resistance cluster with high similarity to corresponding regions in pR478 (Fig. 2). This region is a mosaic composed of a Tn*1696* backbone (with the respective In4 integron) (Partridge et al. 2001) flanked on the right side by the *mer* region of the Tn*21* transposon (Tn*21* protein *urfY2-merEDACPTR*) (Liebert et al. 1999) (Fig.2). The Tn*1696* element had an adjacent outer repeat of 38 bp that was truncated by an IS*4321* element. Multidrug

resistance regions with the same mosaic backbone (*Tn*1696 and Tn*21*) have been found in the FIB plasmid pMRVIM0813 (KP975077) (Fig.2a), and in the untyped plasmid p34983-59 (CP010378) (Fig.2b) of clinical isolates of *Enterobacter cloacae*, suggesting that this resistance module may be frequently transferred as a whole among clinical isolates, eventually of different genera. The In4 variable region of pMRVIM0813 harbors a gene cassette array (*bla*$_{\mathrm{VIM}}$-*aacA4*-*aphA15*-*aadA1*-*catB2*) and a complex transposable region with antibiotic resistance genes inserted before the IS*6100* that is not present in the conjugative plasmidome of strain H1FC54 (Fig.2a). Similarly, in p34983-59 the variable region of In4 contained additional antibiotic resistance genes inserted after the *sulI* gene, also not found in the conjugative plasmidome of strain H1FC54 (Fig.2b). These differences were not unexpected, given the dynamic character of integrons variable regions.

The integron found in the multidrug resistance region of the H1FC54 conjugative plasmidome had characteristics of the In4 type, with an integrase in the 5'-conserved segment (5'-CS), and with genes of resistance to ethidium bromide (*qacEΔ1*) and sulfonamides (*sulI*), a conserved puromycin N-acetyltransferase-like protein (*orfCS*) and a Tn*6100* transposase in the 3'-conserved segment (3'-CS) (Partridge et al. 2001). The variable region of this integron contained four gene cassettes, encoding resistance to fluoroquinolones (*aac(6')-Ib-cr*), to beta-lactams (*bla*$_{\mathrm{OXA\text{-}1}}$), to chloramphenicol (*catB3*) and to rifampicin (*arr3*). Homologous of these integron, with the same gene cassette, have been described in numerous plasmids types and bacterial species recovered from both environmental and clinical isolates (Table 3). In addition to the beta-lactam, chloramphenicol and quinolone resistance determinants associated with the Tn*1696* transposable region, the H1FC54 conjugative plasmidome carried genetic determinants of resistance to these same antibiotics (*bla*$_{\mathrm{SHV\text{-}12}}$, *catA2, qnrB4*) located in other regions of the plasmidome. The genes *bla*$_{\mathrm{SHV\text{-}12}}$ and *catA2* were observed to be associated with IS*26* transposases (Table 3). It was also possible to find in the conjugative plasmidome H1FC54 two toxin-anti-toxin (TA) systems (*hipAB* and *RelE/RelB*) associated with bacterial persistence and multidrug resistance (Chan et al. 2015). Resistance genes detected in the H1FC54 plasmidome have been described in different plasmid types, including of the type HI2, and in a variety of bacterial species recovered from environmental, food production animals and food products and also in clinical isolates (Table 3).

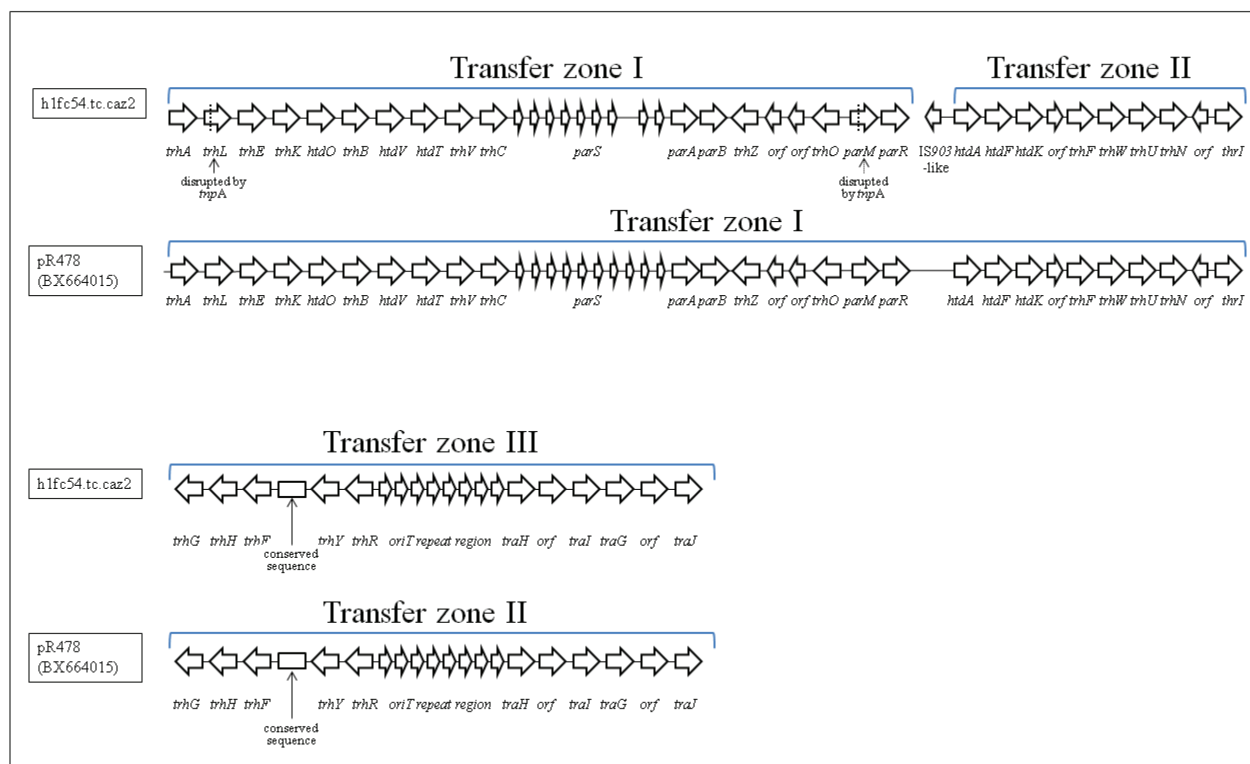

Fig.1 - Structure of the multidrug resistance zone found in the H1FC54 conjugative plasmidome and comparison with analog zones in other plasmids, a) plasmid **pMRVIM0813 (KP975077) from *Enterobacter cloacae*;** b) plasmid **p34983-59 (CP010378) from *Enterobacter cloacae*.** Homologue regions (>99% nucleotide sequence similarity) are underlined;*90%nucleotide sequence similarity; In4 integron, dashed line: Resistance to (fluoro)quinolones: *aac(6')-Ib-cr, qnrB2;* aminoglycosides, *aacA4*, *aac(6')-Ib-cr, aadA1*, *aphA15*; rifampicin, *arr3*; beta-lactams, bla$_{OXA-1}$, *bla*$_{VIM}$; chloramphenicol, *catB2, catB3*; ethidium bromide, *qacEΔ1*; sulfonamides, *sulI*; mercury, *merEDACPTR*; other genes represented: Tn,IS, transposase; TnpR, resolvase; *orfCS*, conserved puromycin N-acetyltransferase-like protein; *intI1*- integrase; TniA, putative transposase; TniB, TniQ, transposase related proteins; *urfY2*, Tn21-associated protein of unknown function;

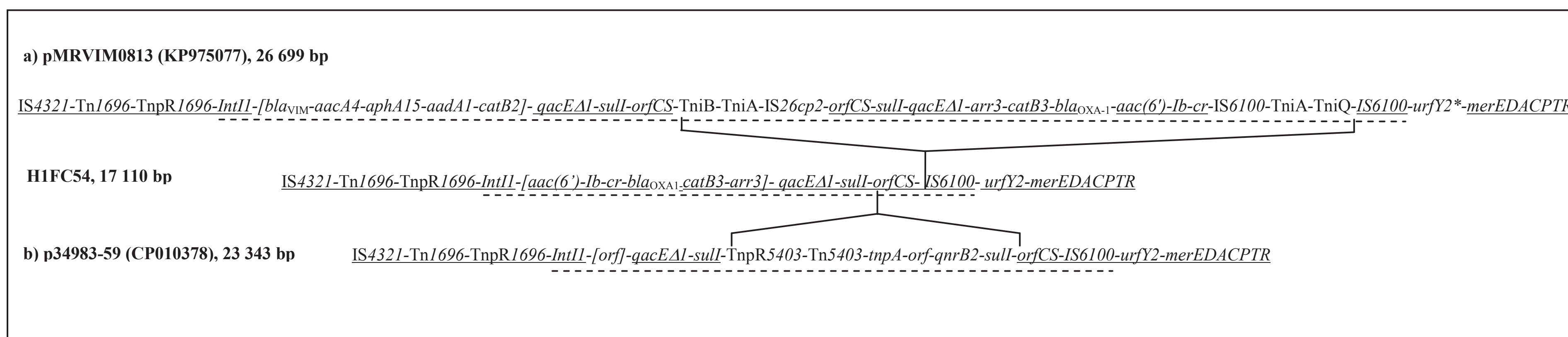

Fig.2 Representation of sequences encoding conjugative transfer functions in the conjugative plasmidome of H1FC54 and in the HI2 prototype plasmid pR478

Table 3 – Genes associated with replication, transfer, gene mobility, resistance and persistence identified in the H1FC54 conjugative plasmidome and examples of closely related genetic elements found in public databases

Function		Gene(s)	Examples of plasmids harbouring regions with sequence similarity >99%[a]	
			inc types	Host strains (Acc.nr)
Replication		*repA*-HI2 *repA*-HIA	plasmids of the same incompatibility group	
Horizontal gene transfer	(region I)	*parR-parM*-trhO-[2 orf]-trhZ-parB-parA-repeat region parS-trhC-trhV-htdT-htdV-trhB-htdO-trhK-trhE-trhL*-trhA*	FIB	*Enterobacter cloacae* (KP975077)
	(region II)	*trhI-[orf]-trhN-trhU-trhW-trhF-[orf]-htdK-htdF-htdA*[+]	HI2	*Enterobacter cloacae* (CP008899, CP008906, EU855787, EU855788), *E.coli* (KT347600[l]), *Klebsiella pneumoniae* (EF382672), *Salmonella enterica* sv. Typhimurium (LN794248, KM877269[l], KT334335, LK056646), sv. Infantis (LN555650[l]) and sv. Heidelberg (JN983042[l]), *Serratia marcescens* (BX664015)
	(region III)	*trh*[a2]*-trhH-trhF-[conserved sequence]-trhY-trhR-oriT repeat region-[orf*[a1]*]-traH-[orf]-traI-traG-[orf]-traJ*	untyped	*Salmonella enterica* sv. Cubana (CP006056[f]), *Enterobacter cloacae* (CP012170)
Antibiotic /metal resistance	Class 1 integron, including genes associated with resistance to aminoglycosides, β-lactams, fluoroquinolones, chloramphenicol, rifampicin, ethidium bromide, and sulfonamides	*Int1*-[*aac(6')-Ib-cr-bla*$_{OXA-1}$*-catB3-arr3*]-*qacEΔ1-sulI-orfCS-IS6100*	FII N U untyped	*Klebsiella oxytoca* (CP011614) *E.coli* (KC960485) *Aeromonas* spp. (NG_040969[r]) *Citrobacter freundii* (CP011611)
	Beta-lactams	Tn*26-bla*$_{SHV-12}$	A/C, A/C+H FIA+R, FIA+X3 HI2 L/M N R X, X3, untyped	uncultured bacterium (KR827393[e]), *Klebsiella oxytoca* (CP003684) *E.coli* (CP010881), *Klebsiella pneumoniae* (CP006801, CP006662) *Enterobacter cloacae* (CP008899, EU855787, EU855788) *Salmonella enterica* sv. Typhimurium (NG_036730) *Klebsiella oxytoca* (HM126016) *Klebsiella pneumoniae* (KF954150) *Enterobacter cloacae* (KR059865), *Klebsiella pneumoniae* (JX104760, KC311431) *Enterobacter cloacae* (CP009857[h], CP012170), *Klebsiella pneumoniae* (CP011983), *Citrobacter freundii* (KP987216)
	Quinolones	*qnrB4*	A/C	*Klebsiella pneumoniae* (LC055503)

	Function	Gene(s)	Examples of plasmids harbouring regions with sequence similarity >99%[a]	
			inc types	Host strains (Acc.nr)
Antibiotic /metal resistance (cont.)			HI	*Klebsiella pneumoniae* (HG918041[ca])
			L/M	*E. coli* (NG_041243)
			R	*Klebsiella pneumoniae* (KF793937)
			untyped	*Citrobacter freundii* (JN215523[w]), *Enterobacter hormaechei* subsp. *oharae* (KF646592), *E.coli* (DQ303921, KC848778[l]), *Klebsiella oxytoca* (NG_036153), *Klebsiella pneumoniae* (NG_036493)
	Tetracycline	*tetA(D)*	A/C, A/C2	*Aeromonas hydrophila* (FJ705807[l]), *Salmonella enterica* sv. Senftenberg (KM670336[l]), *Salmonella typhimurium* (KP276584[l])
			FII	*E.coli* (NC_020086)
			HI2	*Enterobacter cloacae* (EU855787, EU855788)
			untyped	*Citrobacter freundii* (CP011610), *Klebsiella pneumoniae* (CP008930), *Photobacterium damselae* sbsp. *piscicida* (AB277723[l]**),** *Salmonella* spp. (AB089602[l]), *Yersinia pestis* bv. Orientalis (CP000603)
	Chloramphenicol	Tn*26*-*orf*-*catA2*-Tn*26*	FIB	*Salmonella enterica* sv. Enteritidis (CP009767[f])
			FII	*E.coli* (CP000971[e])
			HI2	*Enterobacter cloacae* (CP008899, EU855787, EU855788)
			untyped	*Citrobacter freundii* (CP011611), *Klebsiella pneumoniae* (KP893385, DQ449578, CP011576), *Kluyvera intermedia* (CP011601), *Salmonella* spp. (AB262968[l]), *Salmonella enterica* sv. Schwarzengrund (CP001125[f]) and sv. Choleraesuis (AY509004)
	Mercury compounds	Tn*21* protein *urfY2-merEDACPTR*	A/C, A/C+H	*Klebsiella pneumoniae* (JQ010984), *Klebsiella oxytoca* (CP003684)
			FIA+R, FIB, FII, FII+R	*Klebsiella pneumoniae* (CP008933, CP006662), *Enterobacter cloacae* (KP975077), *E.coli* (KP294351), *Klebsiella oxytoca* (CP009466), *Klebsiella pneumoniae* (CP006657), *Serratia marcescens* (CP011640), uncultured bacterium (KP294350[w])
			H, HI2	*Enterobacter cloacae* (CP008825[h], CP008899, EU855787, EU855788), *Klebsiella pneumoniae* (CP013215, JN420336), *Salmonella enterica* sv. Typhimurium (NG_039598[l], NG_040952[l]), *Serratia marcescens* (BX664015)
			L/M, M, M1	*Klebsiella oxytoca* (KJ541681), *Salmonella enterica* sv. Paratyphi (KM406488) and sv. Typhimurium (KM406490)
			N	*Klebsiella pneumoniae* (GU585907)
			R	*Klebsiella pneumoniae* (KJ958927)
			T	*Citrobacter freundii* (JQ996150)

	Function	Gene(s)	Examples of plasmids harbouring regions with sequence similarity >99%[a]	
			inc types	Host strains (Acc.nr)
Antibiotic /metal resistance (cont.)			untyped	*Citrobacter freundii* (CP011610), *Enterobacter aerogenes* (FO203354), *Klebsiella oxytoca* (CP011634), *Klebsiella pneumoniae* (CP011622), *Kluyvera intermedia* (CP011601), *Pantoea* spp. (CP009869[h]), *Pantoea agglomerans* (NG_034804[wa]), *Salmonella enterica* sv. Cubana (CP006056[f]) and sv. Typhi (AL513383), *Serratia marcescens* (CP011641)
	Tellurite	*terY3Y2XY1W-[6 orf]-terZABCDEF*	FIB	*Enterobacter cloacae* (KP975077)
			HI2, HIA	*Enterobacter cloacae* (CP008825[h], CP008906, CP008899, EU855787, EU855788), *Klebsiella pneumoniae* (EF382672), *Kluyvera intermedia* (CP011601), *Salmonella enterica* sv. Typhimurium (LK056646, LN794248), *Serratia marcescens* (BX664015)
			untyped	*Enterobacter cloacae* (CP012170), *Salmonella enterica* sv. Cubana (CP006057[f])
	Arsenic compounds and sulfate	*tnpA-arsHRBC-sfpBA*	FIB	*Enterobacter cloacae* (KP975077)
			HI1, HI2, HIA	*Citrobacter freundii* (JX182975), *Enterobacter cloacae* (CP008825[h], CP008899, EU855787, EU855788), *Klebsiella pneumoniae* (EF382672), *Salmonella enterica* sv. Typhimurium (LN794248, LK056646) and sv. Infantis (LN555650[l]), *Serratia marcescens* (BX664015)
			untyped	*Enterobacter cloacae* (CP012170), *Klebsiella oxytoca* (CP011617, CP011596), *Klebsiella pneumoniae* (CP007734), *Kluyvera intermedia* (CP011601)
	Cobalt, zinc and cadmium	*tnpA-tnpA-orf-[*Cobalt-Zinc-Cadmium resistance protein]	FIB	*Enterobacter cloacae* (KP975077)
			HI2	*Enterobacter cloacae* (CP008825[h], CP008899, CP008906, EU855787, EU855788), *Klebsiella pneumoniae* (CP013215, EF382672)
			L/M	*Klebsiella pneumoniae* (KC757417)
			untyped	*Enterobacter cloacae* (CP012170), *Kluyvera intermedia* (CP011601)
	Nickel, cobalt and copper	[a2]*rcnR-rcnA-pcoE-pcoS*[+]	FIB	*Enterobacter cloacae* (KP975077)
			HI2	*Enterobacter cloacae* (CP008899, CP008825[h], EU855787, EU855788), *E.coli* (HG530658[l]), *Klebsiella pneumoniae* (EF382672, CP013215), *Salmonella enterica* sv. Infantis LN555650[l])

	Function	Gene(s)	Examples of plasmids harbouring regions with sequence similarity >99%[a]	
			inc types	**Host strains (Acc.nr)**
Antibiotic /metal resistance (cont.)			untyped	*Enterobacter cloacae* (CP012170), *Klebsiella oxytoca* (CP011617, CP011596), *Kluyvera intermedia* (CP011601), *Salmonella enterica* sv. Cubana (CP006057[f])
Persistence	Growth inhibition (Serine-threonine kinase/transcriptional regulator)	*hipA/hipB*	A/C	*E.coli* (KP056256)
			FIB	*Enterobacter cloacae* (KP975077)
			HI2, HIA	*Enterobacter cloacae* (EU855787, EU855788, CP008899, CP008825[h]), *E.coli* (HG530658[f]), *Klebsiella pneumoniae* (EF382672), *Kluyvera intermedia* (CP011601), *Salmonella enterica* sv. Typhimurium (LN794248) and sv. Infantis (LN555650[f])
	Growth inhibition (mRNA interferase/transcriptional regulator)	*relE/relB*	FIB, FII	*Enterobacter cloacae* (CP001919, KP975077), *Salmonella enterica* sv. Choleraesuis (EU219534)
			HI1, HI2	*Citrobacter freundii* (JX182975), *Enterobacter cloacae* (EU855787, EU855788, CP008906, CP008899, CP008825[h]), *Klebsiella pneumoniae* (EF382672), *Kluyvera intermedia* (CP011601)
			L/M	*Klebsiella pneumoniae* (NC_021502)
			N	*Pantoea* spp. (CP009883[h])
			untyped	*Edwardsiella tarda* (HQ332785[f]), *Enterobacter cloacae* (CP012170), *E.coli* (CP006642[f]), *Klebsiella pneumoniae* (CP011623), *Klebsiella oxytoca* (CP011635, CP008843[f]), *Kluyvera intermedia* (CP011600), *Salmonella enterica* sv. Cubana (CP006057[f]) and sv. Schwarzengrund (CP001125[f])

[a]nucleotide sequence similarity values lower than 99 %: [a1]>97% ; [a2]>98%; [+]adjacent to a IS*903*-like transposase; underlined: plasmid pR478 from *Serratia marcescens* (BX664015); *gene disrupted by the insertion of a transposase; The indicated genes and gene clusters were from clinical origin, except when noted: [w] wastewater; [r] river; [l] livestock; [f] food; [ca]companion animals; [h] hospital environment; [e] environment; [wa] wild animal.

Discussion

Due to their ubiquity and relevance for antibiotic resistance dissemination, *Enterobacteriaceae* plasmids are well characterized and classified (Novick 1987). One of such classification systems relies on the definition of plasmid incompatibility groups in *Enterobacteriaceae* (Couturier et al. 1988). Plasmids of the replicon type HI2 are amongst the incompatibility plasmids groups early identified in *Enterobacteriaceae* (Couturier et al. 1988) and have been described as relevant carriers of genes encoding resistance to drugs such as third generation cephalosporins, carbapenems, aminoglycosides, sulfonamides and tetracyclines (Elhani et al. 2010; Coelho et al. 2012; Wong et al. 2014; Liu et al. 2015). The mobility of plasmids of the HI2 type among *Enterobacteriaceae* spans a wide distribution across distinct bacterial genera and species, including *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica* and *Enterobacter cloacae,* and has been documented in several studies (Liu et al. 2013; Nilsen et al. 2013). Although the presence of HI2 type plasmids in wastewater and associated environments has been reported (Becerra-Castro et al. 2015), extensive nucleotide sequence characterization has been performed only for isolates associated with the clinical practice, with animal pathologies or food outbreaks (Gilmour et al. 2004; Johnson et al. 2006; Chen et al. 2007; Chen et al. 2009; Han et al. 2012; Conlan et al. 2014; Feasey et al. 2014; Falgenhauer et al. 2015; Kariuki et al. 2015). The characterization of HI2 plasmids from wastewater isolates has been based in gene-targeted methods (e.g. PCR) (Becerra-Castro et al. 2015; Varela et al. 2015; Vaz-Moreira et al. 2015), but this type of approach may neglect the presence of important genetic determinants associated with resistance and gene mobility.

The analysis of the H1FC54 conjugative plasmidome suggested the occurrence multiple events of gene recombination, either at the cellular or molecular levels. Although the backbone regions encoding for conjugation and transfer in H1FC54 shared high nucleotide sequence similarity with the prototype HI2 plasmid pR478, the presence of sequences with high similarity with other plasmids from diverse taxonomic groups and origins suggested the strong plasticity of the H1FC54 conjugative plasmidome. A similar finding was reported by Veldman et al. (2010), who analyzed the nucleotide sequence a HI2 plasmid from *Salmonella* Concord. The authors found that although the analyzed plasmid could be classified as pR478-like, it carried determinants of antibiotic resistance that were related to the plasmid pAPEC-O1 (DQ517526) (Johnson et al. 2006). Indeed, the

backbone of HI2 plasmids is known for its plasticity, harboring genes that are typically found in clinically relevant isolates, associated with humans. (e.g. Chen et al. 2007; Kariuki et al. 2015). The presence in the conjugative plasmidome H1FC54 of genes and gene clusters with high nucleotide sequence similarity to a diverse set of bacterial genera highlights the potential of this plasmid backbone for the acquisition and dissemination of DNA elements from the bacterial gene pool through horizontal gene transfer. Transfer of the HI2 plasmid under study was shown to supply determinants of resistance to antibiotics such as beta-lactams, sulfonamides, aminoglycosides, rifampicin, aminoglycosides, (fluoro)quinolones and tetracycline to the receptor bacteria. Transfer of determinants conferring resistance to these classes of antibiotics due to HI2 transfer by conjugation is not uncommon and has been previously reported in hospital effluent (Vaz-Moreira et al. 2015), in isolates from poultry (Liu et al. 2013) and in clinical isolates (Coelho et al. 2012).

The ability of HI2 plasmids to be transferred *in vitro* from clinical isolates of *E.coli* to the azide-resistant *E.coli* J53, in the absence of antibiotics, has been reported previously, with conjugation rates in the orders of 10^{-9} to 10^{-3} at 25°C (García et al. 2007). The conjugation efficiencies observed in this study (10^{-8} to 10^{-5}, in the absence of antibiotics) differed slightly from those reported in the literature, which we speculate may be related the method used, of the donor strain and of the plasmids being transferred.

In this study, the presence of sub-inhibitory concentrations of antibiotics was observed to influence the rate of conjugation, being apparently stimulated in the presence of ceftazidime and inhibited in the presence of tetracycline. Probably due to the antagonist effects, when both antibiotics were present the rate of conjugation was not significantly different from the antibiotic-free conditions. The existence of a correlation between the exposure to sub-inhibitory concentrations of antibiotic and the increase in conjugation rates has been discussed in the literature (Andersson & Hughes 2014). Zhang et al., (2013) demonstrated that the combination of kanamycin and streptomycin, but not these antibiotics alone or other combinations of antibiotics, increased the conjugation of the plasmids pRK2013, pSU2007, or RP4 between *E.coli*. The authors attributed the effect to an upregulation of expression of conjugation-associated genes in the presence of those antibiotics. A similar effect was observed in the presence of sub-inhibitory concentrations of tetracycline during conjugation by Kim et al. (2014), who reported the impact of these conditions on the increased transfer frequencies of the plasmid pB10 from *E.coli* DH5α to an activated sludge bacterial community. The *in vivo* expression of genes mediating conjugative transfer among *Aeromonas hydrophila* of the plasmid pRAS1 (which carries

determinants of resistance to tetracycline, trimethoprim and sulphonamide) was shown to be enhanced in the presence of sub-inhibitory concentrations of tetracycline and trimethoprim (Cantas et al. 2012). However, the exact cellular mechanisms behind the effect of sub-inhibitory concentrations of antibiotics in the conjugative transfer of plasmids are still unclear. We hypothesize that the increase in conjugation efficiency we observed in the presence of ceftazidime, as well as the decreased conjugation rates associated with tetracycline, are the consequence of the effect of these antibiotics in the conjugation mechanisms.

As expected given the fact that transconjugants were recovered on culture medium supplemented with ceftazidime, all transconjugants presented an increased MIC_{CAZ} value and acquired the genes *bla*$_{SHV-12}$ gene and *bla*$_{OXA-1.}$ These results are in line with those reported for the first identification of *bla*$_{SHV-12}$ in clinical isolates *of Klebsiella pneumoniae* (Nüesch-Inderbinen et al. 1997). These authors demonstrated that although the acquisition of the *bla*$_{SHV-12}$ gene by transconjugants was associated with an increase in the MIC_{CAZ}, the increase in resistance was not similar for all transconjugants acquiring the gene (with the MIC_{CAZ} ranging 32-64 μg/ml). This suggests that the presence of this gene alone is not responsible for a fixed ceftazidime resistance phenotype.

An additional consequence of the positive selection of antibiotic resistante strains caused by the presence of antibiotics is the co-selection of unrelated genes encoded in the genome of the surviving bacteria, such as determinants of resistance to other antibiotics, disinfectants, metals and other toxic compounds. In this study the association between antibiotic and metal resistance genes was evidenced by the fact that both types of determinants were transferred by conjugation. Additionally, it was possible to find determinants of resistance to antibiotics and metals inserted in the same mobile multidrug resistance region (Tn*1696*-Tn*21*), suggesting a strong association and the potential for co-mobilization of these genes.

In addition to determinants directly implicated in resistance mechanisms, the H1FC54 conjugative plasmidome was found to carry the toxin-antitoxin systems *hipAB* and *RelE/RelB*, which have been associated with antibiotic tolerance in bacterial populations due to the formation of persister cells (Maisonneuve et al. 2011). The presence of *Rel//RelB* in particular can be an additional factor contributing to the resistance of the isolate H1FC54 to fluoroquinolones and beta-lactams, as the production of *RelE* has been shown to increase in bacterial populations exposed to the ciprofloxacin or ampicillin

(Maisonneuve et al. 2011). The presence of the *RelE/RelB* and *hipAB* in the H1FC54 plasmidome, together with the clusters of genes that encode for conjugation and transfer systems, may ensure that the antibiotic and metal resistance genes encoded in H1FC54 plasmids can both persist in the bacterial population during the exposition to antibiotics and toxic metal compounds, and be transmitted to other bacteria.

Overall, the analysis of the H1FC54 conjugative plasmidome illustrates the potential of bacteria from hospital effluent to disseminate genetic determinants of resistance to antibiotics, metals and toxic compounds. Variations in the conjugation rates observed in the presence of sub-inhibitory concentrations of antibiotics show how these conditions may enhance the rate of plasmid transfer. The plasticity of the conjugative plasmidome of H1FC54 is evident from its mosaic structure, constituted from a HI2 backbone shaped by events of gene acquisition and recombination. The sequences under analysis were shown to gather genetic determinants shared with bacteria from both clinical and environmental sources, which hints at the role of this type of plasmid in the circulation of determinants of resistance between these habitats. The potential for bacteria from hospital effluents to participate in the dissemination of resistance is stressed.

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General conclusions

The rise in bacterial antibiotic resistance over the last decades has become an issue of growing concern (Laxminarayan et al. 2013; World Health Organization 2014). The increase in resistance prevalence has been largely attributed to the overuse of antibiotics as therapeutic agents (Davies & Davies 2010; Van Boeckel et al. 2014). The presence of antibiotics constitutes a selective pressure which favors the survival of strains that harbor resistance determinants over susceptible ones, creating the ideal context for the proliferation of resistant lineages (Oz et al. 2014). Although the therapeutic use of antibiotics has a recognized responsibility in the development of bacterial resistance, the effect of the release of antibiotic residues into the environment can not be disregarded (Berendonk et al. 2015). Environmental reservoirs of antibiotic resistant bacteria derived from anthropogenic activities include, for example, aquatic environments such as wastewater, sewage resulting from industrial facilities such as pharmaceutical plants, aquaculture or livestock barns (e.g. Fick et al. 2009; Mokracka et al. 2012), and lands fertilized with active sludge resulting from the wastewater treatment process (Rahube et al. 2014). Water, in particular, has a privileged role in the dissemination of antibiotic resistant bacteria, as it permeates most environmental compartments. The study of the environmental dissemination of bacterial antibiotic resistance is made through the analysis of community structure (e.g. Vaz-Moreira et al. 2012; Novo et al. 2013), of resistance prevalence values (e.g. Figueira et al. 2012; Odjadjare et al. 2012) and of genetic determinants of antibiotic resistance (e.g. Figueira et al. 2011; Narciso-da-Rocha et al. 2014) over different compartments.

Wastewater aggregates antibiotic residues together with antibiotic resistant bacteria, antibiotic resistance genes and associated mobile genetic elements, creating the conditions that favor the selection of resistant bacteria and the exchange of genetic determinants of resistance between them (Szczepanowski et al. 2009; Moura et al. 2012; Novo et al. 2013). Hospital effluents, in particular, are privileged recipients of antibiotic resistant bacteria from clinical activities, and of antibiotic residues in concentrations higher than those found in ordinary municipal effluents (Kümmerer & Henninger 2003; Verlicchi et al. 2010). Nevertheless, in most world regions, hospital effluents are classified as domestic effluents and are not required to undergo any dedicated treatment before being discharged into municipal collectors (Carraro et al. 2016). The risk for the emergence and dissemination of

antibiotic resistant bacteria and their genes in this type of environment is thus potentially elevated. Therefore, the analysis of the variations suffered by bacterial communities in wastewater in the presence of antibiotic residues was considered as a starting point for this study (Chapter 1).

An analysis of the variation of the bacterial communities from hospital effluent and from the urban wastewater treatment plant receiving those effluents revealed the existence of correlations between the variation of the concentration of antibiotics and metals and the relative abundance of some bacterial populations (supposedly, each represented by a DGGE band), suggesting that the presence of antibiotic residues or metals may be, somehow, related with rearrangements of the bacterial communities in wastewater. The hospital effluent under study differed from the raw inflow and the treated effluent of the receiving urban wastewater treatment plant on the concentration of antibiotics and metal residues, with different contaminants peaking in hospital effluent (ciprofloxacin and ofloxacin, mercury and arsenic) or in the untreated municipal effluent (sulfamethoxazole, tetracycline) . These differences seemed to coincide with the presence of distinct antibiotic resistant populations in each type of wastewater, with ciprofloxacin resistant bacteria being more prevalent in hospital effluent than in the urban wastewater treatment plant. Indeed, it was possible to observe a positive correlation, especially in hospital effluent samples, between variations of the presence of ciprofloxacin resistant populations (heterotrophs, enterobacteria, aeromonads and pseudomonads) and the concentration of ciprofloxacin residues. Additionally, it was also possible to observe positive correlations between the variations of the amoxicillin (beta-lactam) resistant aeromonads and pseudomonads and the concentration of penicillin G (beta-lactam) and tetracycline residues for the generality of wastewater samples. Overall, these results are in line with previous studies that documented the existence of a correlation between variations of antibiotic or metal concentrations in wastewater and of the antibiotic resistant bacterial populations has been previously documented (Huerta et al. 2013; Novo et al. 2013), and generally point towards a the existence of a relationship between the presence of residues and the increase in the prevalence of resistance. Novo et al. (2013) reported a correlation between variations in the concentration of tetracyclines in untreated effluent and in the prevalence of antibiotic resistant populations, although not specifically tetracycline resistant, in the treated effluent of the same wastewater treatment plant. Huerta et al. (2013) reported positive correlations between the concentration of macrolides and the determinant of resistance to macrolides *ermB* in artificial water reservoirs with wastewater treatment plant effluent input.

Similarly, the occurrence of significantly higher ($\alpha<0.05$) values of prevalence of antibiotic resistant bacteria and antibiotic resistance genes in hospital effluent than in municipal wastewater has also been previously documented in studies focusing on ciprofloxacin and vancomycin resistant enterococci (Varela et al. 2013), or on genetic determinants of resistance to vancomycin (*vanA*), to beta-lactams (bla_{TEM}) and of multiresistance (*marA*) (Narciso-da-Rocha et al. 2014). Together with the results of the present study, this suggests a potential impact of hospital effluents in the levels of antibiotic resistant bacteria in municipal collectors. Indeed, such impact has been assessed in studies such as Harris et al. (2013), which documents the occurrence of comparatively higher values of prevalence of ciprofloxacin, tetracycline and sulfonamide resistant bacteria in a wastewater treatment plant that receives hospital effluent than in an urban wastewater treatment plant without such type of input. The results from the present study and from other authors (Ferreira da Silva et al. 2006; Ferreira da Silva et al. 2007; Novo et al. 2013) demonstrate that although the effective number of bacteria in wastewater is decreased by the wastewater treatment, a corresponding reduction is not observed for the levels of antibiotic resistance prevalence. Hence, it can be hypothesized that the input of hospital effluent in municipal wastewater may also impact the environments that, directly or indirectly, receive the discharge of the wastewater treatment plant.

The observation that a well-functioning urban wastewater treatment plant discharges on average 10^2 to 10^4 antibiotic resistant bacteria per milliliter motivated further research to investigate if bacteria present in wastewater were spread in other environmental compartments. Therefore, the next stage of this study was the tracking of selected bacterial lineages across different sites to assess if they could have a role in the dissemination of antibiotic resistance. The rationale was the cross checking of data regarding the genetic variability of bacterial hosts and their antibiotic resistance determinants and associated mobile genetic elements. The aim was at inferring simultaneously about the vertical transmission (based on chromosomal genes) and the horizontal transfer (based on acquired determinants) of resistance.

Given its importance as indicator of fecal contamination and vector of antibiotic resistance, *Escherichia coli* was selected as a model species for this study. A collection of strains with reduced susceptibility to fluoroquinolone, isolated from sampling events spanning nine years, was characterized in terms of genetic diversity (based on the nucleotide sequence of housekeeping genes), prevailing mechanisms of quinolone

resistance and for the presence of additional genetic elements associated with antibiotic resistance.

E.coli isolates genotyped based on multilocus-sequence typing (MLST) from different dates, conditions of isolation and water environments (hospital effluent, urban wastewater treatment plant raw inflow and treated effluent, and urban streams), were observed to cluster together, strongly suggesting the existence of *E. coli* lineages that are ubiquitous and have the potential to persist in water environments over time. The most prevalent lineages belonged to ST131 and to the ST10 complex, described in the literature as ubiquitous but also frequently associated with humans and other animals in a clinical or veterinary context (Valverde et al. 2009; Peirano et al. 2012; Nicolas-Chanoine et al. 2013; Nicolas-Chanoine et al. 2014). Among less represented sequence types it was also possible to find lineages that have been reported in pets and food production animals (e.g. ST533, ST648, ST744, ST224) (Hasan et al. 2012; Huber et al. 2013; Pires-dos-Santos et al. 2013; Schink et al. 2013; Wagner et al. 2014), which evidences the potential of these bacteria to circulate over different hosts and environmental compartments. Some of the identified lineages have been recognized for their role in antibiotic resistance propagation in clinical or veterinary settings, and are associated with the dissemination of acquired quinolone and beta-lactam resistance (eg. Jones et al. 2008; Valverde et al. 2009) and of virulence determinants (Mellmann et al. 2008; Wagner et al. 2014), which suggests the role that wastewater may have in the circulation of bacteria of clinical and/or veterinary relevance. The finding that the antibiotic resistance phenotypes of some isolates could be partially attributed to the presence of acquired genetic determinants of resistance further reinforces this idea. Indeed, while most of the phenotypes of resistance to quinolones identified in this group of *E.coli* seemed to be due to point mutations in the quinolone resistance-determining regions (QRDR) of its target enzymes (DNA gyrase and topoisomerase IV), it was also possible to identify determinants of resistance to quinolones typically associated to mobile genetic elements, more specifically the plasmid mediated quinolone resistance (PMQR) genes *qnrS1* and *aac(6')-Ib-cr*. The occurrence of acquired quinolone resistance genes was not observed to be associated with any particular bacterial lineage; however, it was related to the origin of the isolates, *i.e.* hospital effluent and untreated effluent, or the isolation conditions, *i.e.* in bacteria isolated on ciprofloxacin-supplemented culture medium, as previously documented in previous studies on quinolone resistant *E. coli* (Figueira et al. 2011b; Vredenburg et al. 2013). Although the resistance level conferred by the PMQR genes found (*qnrS1* and *aac(6')-Ib-cr)* is reportedly low, it has been suggested

that their presence may serve as a background to the development of higher resistance levels as it increases the sub-inhibitory concentration of antibiotic tolerated by the bacterial population, and therefore maximizes the opportunity for mutant selection (Jacoby 2005; Drlica & Zhao 2007). The finding that isolates from hospital effluent and from the untreated effluent to the urban wastewater treatment plant carry genes conferring resistance to sub-inhibitory levels of ciprofloxacin may partially explain the correlation previously observed between sub-inhibitory concentrations of ciprofloxacin and ciprofloxacin resistant bacteria (Varela et al. 2014). Interestingly, these determinants were only detected in raw effluents (from hospital and municipal treatment plants), where the selective pressure from the presence of quinolones was found to be comparatively higher than in the treated effluent. The occurrence of antibiotic resistance-conferring mutations is thought to be facilitated in the presence of quinolones, as these antibiotics have been shown to trigger cellular responses which includes both the overexpression of selected PMQR genes (Da Re et al. 2009) and an increase in mutation rates that results in the emergence of high-level quinolone resistance associated point mutations in the QRDR (Andersson & Hughes 2012). The absence of PMQR in isolates from treated effluent may also be attributed to plasmid loss, which has been shown to occur in the absence of selective pressure (Smith & Bidochka 1998).

In general, the presence of PMQR in an isolate was also associated with beta-lactam resistance genes, reinforcing the hypothesis of co-selection of beta-lactam and quinolone resistance determinants that arose from the analysis of the ceftazidime resistance prevalence values for isolates recovered in ciprofloxacin-supplemented culture media. Co-selection of resistance to quinolone and beta-lactams has been previously reported in patients undergoing treatment with either of the antibiotics and constitutes a risk factor for the emergence of multidrug resistant and ESBL-producing isolates (Wener et al. 2010; Cao et al. 2011). Also of notice was the association of PMQR genes with several different plasmid replicon types, indicative of the high potential of these genes to be mobilized.

The potential for antibiotic resistance transmission from these bacteria was evidenced by conjugation experiments, in which resistance to sulfamethoxazole, to amoxicillin/ticarcillin and to tetracycline could be acquired by transconjugants. Plasmids of different incompatibility groups (FIA, FII, I1-Iγ, K, B/O, HI2 and N), beta-lactam resistance genes bla_{TEM}, bla_{SHV} and bla_{OXA}, and PMQR genes *qnrS1* and *aac(6')-Ib-cr* could be horizontally transferred. Co-transference of genetic determinants of resistance to beta-

lactams and quinolones was observed in several occasions, reinforcing the previous hypothesis of co-selection of beta-lactam and quinolone resistance.

Once assessed the wide dissemination of antibiotic resistant *E. coli* lineages in different water compartments, it was considered a priority the evaluation of the relationship with human-clinical and wild-life isolates.

The comparison of water quinolone-resistant *E. coli* isolates with others from hospitalized patients, urban gulls and birds of the prey (living in a natural reserve), based on MLST revealed that, while birds of the prey generally host lineages distinct of those isolated from humans and human-impacted environments, isolates from all the other sources were found to cluster together. Isolates originating from wastewater, urban streams and urban gulls, in particular, presented a homogeneous distribution and could be found clustered with isolates from either patients or wild birds. Indeed, the groups of isolates from wastewater and urban gulls presented the higher diversity and richness and higher evenness indices than the other groups of isolates under study, suggesting a higher diversity of bacterial input for these sources. The potential of hospital effluent and of urban birds to serve as a vehicle for the dissemination of antibiotic resistance from the clinical settings into environmental compartments is suggested by the fact that these were the reservoirs where highest multidrug resistance indices were found (≥MDR4, resistance to antibiotics from over four distinct classes).

Similarly to what had been observed for water isolates, high levels of quinolone resistance in isolates from birds of the prey, gulls and clinical isolates were associated primarily with chromosomal mutations, but low-level resistance associated genes were also present. In addition to water isolates, the gene *aac(6')-Ib-cr* was also present in isolates from gulls and patients, while the gene *qnrB* was found exclusively in isolates from clinical origin. The recovery of the same PMQR genes from isolates of different bacterial lineages and origins highlights their potential for horizontal transfer, which as discussed above may have a role in the spread of antimicrobial resistance. These findings suggest the existence of pathways of dissemination between these different settings. The high mobility that both gulls and wastewater have in the environment makes them prone to contact with a wide range of bacterial sources. It also means that both wastewater and urban birds may be important vectors of dissemination of antimicrobial resistance in the environment. Although the study of the dissemination of antibiotic resistance has been preferentially focused in clinically relevant bacteria, there is also a growing concern over environmental bacteria that may have the potential to act as reservoirs of antibiotic resistance genetic

determinants. In order to approach this aspect of the antibiotic resistance dissemination, and supported by the finding of a correlation between the presence quinolone resistant *Aeromonas* spp. and of the concentration of fluoroquinolones during the 16S-rRNA DGGE-based study of bacterial communities (Varela et al. 2014), this genus seemed worthy of further investigation

Although *Aeromonas* spp. are not in the list of the leading agents of health care associated infections (European Centre For Disease Prevention and Control 2012), they are known for its role as important antibiotic resistance reservoirs in water environments (Janda & Abbott 2010; Piotrowska & Popowska 2014). Therefore, a set of *Aeromonas* isolates from hospital effluent, raw inflow and treated wastewater of the receiving urban wastewater treatment plant was selected for characterization. As observed for *E.coli*, quinolone resistant *Aeromonas* spp. presented high prevalence of resistance to amoxicillin, ticarcillin and multidrug resistance (MDR4), with resistance to ceftazidime among the least prevalent phenotypes, and being observed mainly among hospital effluent isolates. Resistance to meropenem and colistin had very low or null prevalence in these bacterial groups, *Aeromonas* and *E.coli*, respectively. For *Aeromonas*, however, and contrary to what was observed for *E.coli*, the percentage of MDR in hospital effluent was significantly higher than in municipal wastewater.

The analysis of acquired quinolone resistance in *Aeromonas* revealed that the presence of the PMQR gene *aac(6')-Ib-cr*, often in combination with *qnrS2* was significantly ($\alpha<0.05$) more prevalent among isolates recovered on GSP supplemented with ciprofloxacin. In *Aeromonas*, the gene *aac(6')-Ib-cr* was associated to MDR phenotypes, suggesting the presence of this gene as a potential indicator of antibiotic resistance dissemination. The gene *qnrS2*, on the contrary, was significantly ($\alpha<0.05$) more frequent in isolates with resistance to less than four distinct classes of antibiotics. While both genes confer low-level resistance to quinolones, it was suggested that their dissemination may involve distinct routes (e.g. in different mobile genetic elements, with distinct properties). Indeed, while the gene *aac(6')-Ib-cr* was common in isolates from both hospital effluent and the urban wastewater treatment plant, and had also been found in *E.coli*, the gene *qnrS2*, which was frequently associated with the plasmid replicon incU, had notoriously a non-clinical origin and was found in *Aeromonas* spp. but not in *E.coli.* This reinforces the suggestion that these genes have an independent circulation, as previously proposed by Park et al. (2006) and Guillard et al. (2014). Hinting the importance of *Aeromonas* spp. as environmental vectors of antibiotic resistance genes,

isolates from treated effluent were observed to hold PMQR genes, something that was not evident in *E. coli*-based studies. Similarly to what was observed for *E.coli*, most isolates with PMQR, and in particular with the gene *aac(6')-Ib-cr*, were also found to harbor determinants of resistance to beta-lactams. The association between both types of determinants is not uncommon and has been reported before for *Aeromonas* by Marti & Balcázar (2012) and Picão et al. (2008). In this study, it was also possible to identify a determinant of beta-lactam resistance ($bla_{OXA\text{-}101}$) that had not been described either in members of the genus *Aeromonas* or in environmental isolates (Juan et al. 2010; Martinez et al. 2014). This evidences the potential for interspecies antibiotic resistance dissemination, and the role of *Aeromonas* spp. as reservoirs of antibiotic resistance determinants from the clinical environments. In addition, as hypothesized initially, it was confirmed that *Aeromonas* may constitute an alternative route to *E.coli* for the dissemination of genetic determinants of resistance in the environment.

One emerging concern in the discussion of the spread of antibiotic resistance in the environment refers to the potential of sub-inhibitory concentrations of antibiotics to stimulate horizontal gene transfer of antibiotic resistance genes (Andersson & Hughes 2014). Once studied the effect of antibiotics concentrations on the wastewater bacterial community and antibiotic resistant population (Varela et al., 2014), it seemed pertinent to investigate the role of the presence of antibiotics on *in-vitro* conjugation events. With this aim, an MDR6 isolate from hospital effluent, previously characterized for its ability to transfer antibiotic resistance genes and a putative broad-host range HI2 plasmid through conjugation (Varela et al. 2015), was selected for further studies.

The presence of sub-inhibitory concentrations of antibiotics was observed to influence the rate of conjugation from the selected isolate, either significantly ($\alpha<0.05$) increasing (ceftazidime) or decreasing (tetracycline) conjugation rates. Curiously, in antibiotic-free conditions and in the presence of both antibiotics the rate of conjugation was not significantly different, suggesting the existence of an antagonist effect from both antibiotics, whose mechanism, nevertheless, could not be explained. Besides the confirmation that sub-inhibitory concentrations of ceftazidime could enhance the conjugation rate, the co-transference of resistance to beta-lactams, sulfonamides, tetracycline, and an increase in the minimum inhibitory concentration of ceftazidime and ciprofloxacin, associated with the acquisition of HI2 plasmid replicon together with determinants of resistance to the respective antibiotics were demonstrated. In order to shed

additional light on the processes of resistance dissemination and co-selection events, the nucleotide sequence of the conjugative plasmidome was further analyzed.

The conjugative plasmidome was observed to contain determinants of resistance to beta-lactams, quinolones, tetracycline, sulfonamides and other antibiotics, as well as to metals and metalloids, such as mercury and arsenic compounds, and transposon regions. The presence of these determinants of resistance may facilitate the survival of the donor strain in some environmental conditions (e.g. hospital effluent), which had been previously shown (Chapter 1) to be contaminated with mercury, arsenic, beta-lactams, sulfonamides, tetracycline and quinolones. Genes encoding resistance to mercury were found to be incorporated in the same transposon as genes of resistance to beta-lactams, fluoroquinolones, chloramphenicol, rifampicin, sulfonamides and ethidium bromide. Curiously, it had been previously observed (Chapter 1) that the concentration of arsenic was, together with that of ciprofloxacin, significantly correlated with the prevalence of quinolone resistant enterobacteria. The recovery of a multidrug resistant isolate with genes of resistance to mercury and arsenic compounds inserted in conjugative plasmids and which transfer may be stimulated by sub-inhibitory concentrations of antibiotic, suggests that both metal and antibiotic residues may have a role on the persistence and dissemination of antibiotic resistance genetic determinants in a microbial community.
Overall, the results obtained in this study suggest that

1. As hypothesized hospital effluent was observed to be a relevant, although not unique, source of antibiotic residues and metals and antibiotic resistant bacteria and genes in municipal wastewater treatment plants.
2. The presence of antibiotic residues in wastewater was correlated with bacterial community rearrangements and with increases in the prevalence of antibiotic resistant bacteria, being this effect more evident in hospital effluent.
3. Municipal wastewater, and in particular those receiving hospital effluents, may constitute a potential reservoir for the dissemination of antibiotic resistant bacteria and antibiotic resistance genes associated with human and animal commensal bacteria into the environment.
4. Ubiquitous bacterial lineages, particularly those that have been associated with humans and other animals and that also have the ability to survive in a variety of environmental

compartments, may serve as carriers of antibiotic resistance genes between humans and the environment.

5. Environmental bacteria such as *Aeromonas* spp., which are not frequently commensal or pathogens in human and other animals, may have a role in the dissemination of antibiotic resistance in the environment.
6. Sub-inhibitory concentrations of antibiotics may influence the rate of transfer of antibiotic resistance genes between bacteria, sometimes enhancing the dissemination of antibiotic resistance to antibiotics and to metals as well as other traits, such as those that may favor the survival and persistence in the environment.

Proposals for future work

The present study aimed at contributing to improve the understanding of the ecology of antibiotic resistance in wastewater environments, seeking for an integrated approach, where antibiotics and metals as environmental contaminants, bacterial communities, bacterial species and lineages and antibiotic resistance and other genetic determinants were analyzed in parallel. The initial hypothesis, that hospital effluent could play a specific role in the emergence and dissemination of antibiotic resistance into the environment, was supported by phenotypic and genotypic data. Throughout the completion of the study, it became apparent that many other questions could be addressed in order to enrich this body of knowledge and aid with decision-making processes that concern water quality improvement. Possible approaches in order to answer to these questions include:

- A broader characterization of the sources of antibiotic resistance into the environment (clinical settings, veterinary practice, domestic effluents, aquaculture systems, industrial sewage) regarding the presence of bacterial groups and genetic determinants of antibiotic resistance. These might be relevant contributions to implement and/or improve the wastewater treatment technologies available, mitigating the release of antibiotic resistance to the environment.

- The further study of the pathways of dissemination of antibiotic resistant bacteria, from the clinical and veterinary settings into the environment via wastewater, with focus on the circumstances that may increase (e.g. biofilm formation in water pipes, presence of sub-inhibitory concentrations of antibiotic) or decrease (e.g. separation of patients fecal residues, preliminary dedicated wastewater treatment) the amount of antibiotic resistance genetic determinants that reach the environment. This type of knowledge could be useful to support decision-making processes in public health and environmental engineering and in hazard analysis and critical control points (HACCP) plans.

- Further characterization of bacterial groups that may act as carriers of antibiotic resistance (e.g. *Aeromonas* spp., *Acinetobacter* spp.,), and of antibiotic resistance determinants and associated genes related to resistance dissemination from the clinical settings (e.g. extended spectrum beta-lactamase, integron and plasmid genes), in order to establish the basis for antibiotic resistance surveillance in environmental

compartments (for example with the use of metagenomic tools) and to define parameters for a safer release of effluents;

- Study of the mechanisms that underlie the increase of antibiotic resistance determinant transfer under the influence of antibiotics, by real-time tracking of gene expression in selected circumstances, in order to develop strategies of wastewater treatment that will minimize antibiotic resistance gene transfer among bacteria.

- Identification of other relevant environmental reservoirs of antibiotic resistance (e.g. urban birds), in order to better contain antibiotic resistance dissemination from such sources and also minimize exposure of humans and animals.

References

(Introduction and General conclusions)

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