Comparison of ubiquitous antibiotic-resistant *Enterobacteriaceae* populations isolated from wastewaters, surface waters and drinking waters

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ABSTRACT

This study aimed at assessing the role of ubiquitous (non-*Escherichia coli*) *Enterobacteriaceae* in the dissemination of antimicrobial resistance through the urban water cycle. *Enterobacteriaceae* isolated from a municipal wastewater treatment plant (111 isolates), urban water streams (33 isolates) and drinking water (123 isolates) were compared in terms of: (i) genera distribution, (ii) resistance to 12 antibiotics, and (iii) class 1 and class 2 integrons. The predominant bacterial genera were the same in the different types of water, although with a distinct pattern of species. The most prevalent resistance phenotypes were observed for amoxicillin, ticarcillin, cephalothin and sulphamethoxazole (24–59% in the three types of water). No resistance against ceftazidime or meropenem was observed. Resistance to cephalothin, amoxicillin and sulphamethoxazole was significantly more prevalent in drinking water, water streams and wastewater, respectively, than in the other types of water. It was possible to recognize antibiotic-resistance associations, namely for the pairs streptomycin–tetracycline (positive) and ticarcillin–cephalotin (negative). Class 1 and/or class 2 integrons with similar gene cassettes were detected in the three types of water. This study demonstrated that *Enterobacteriaceae* are important vehicles of antibiotic resistance, namely in drinking water.

Key words | antibiotic resistance, class 1 integron, class 2 integron, drinking water, fecal contamination, ubiquitous *Enterobacteriaceae*

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INTRODUCTION

Over the last years the epidemiology of antibiotic-resistant *Enterobacteriaceae* had a significant change (Denton 2007). Antibiotic-resistance prevalence has increased and new resistance genotypes, mainly against beta-lactams, have emerged. Frequently, such resistance genotypes rapidly disseminate to the community (Livermore & Woodford 2006; Paterson 2006; Denton 2007; Gould 2008). In part, such changes may be due to the ability that bacteria have to move between different environmental niches and to promote horizontal gene transfer processes (Baquero *et al.* 2008; Martinez 2009). In this respect, bacteria that can colonize humans and other animals and survive in the environment, for example in aquatic systems, have been regarded as important vectors of antibiotic-resistance dissemination (Houndt & Ochman 2000; Baquero *et al.* 2008; Martinez 2009). In particular, any source of human-derived fecal contamination, which may include antibiotic-resistant organisms and/or substances with antimicrobial activity, is considered an important reservoir of antibiotic resistance (Baquero *et al.* 2008; Kümmerer 2009a, b).

The family *Enterobacteriaceae* comprises different genera and species, which according to their ecology and preferential habitats were tentatively divided into different groups – fecal (e.g. *Escherichia coli*), ubiquitous (e.g.

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Citrobacter, *Klebsiella* or *Enterobacter*) and environmental (e.g. *Raoultella* or *Buttiauxella*) (Leclerc *et al.* 2001). Among these, *E. Coli* members are considered the most reliable indicators of fecal contamination and are used to assess microbiological water quality and safety (Council Directive 98/83/EC 1998; Edberg *et al.* 2000; Leclerc *et al.* 2001; WHO 2004). Mainly for that reason, the majority of the studies and reviews focusing antibiotic resistance in environmental *Enterobacteriaceae* give special emphasis to *E. Coli* (Goñi-Urriza *et al.* 2000; Ferreira da Silva *et al.* 2007; Watkinson *et al.* 2007; Laroche *et al.* 2009; Kümmerer 2009b; Martinez 2009). Nevertheless, if the ecology and physiology of *Enterobacteriaceae* is taken into account, it becomes evident that not only *E. Coli*, but many other members of the same family, can be important vehicles of antibiotic-resistance dissemination. For example, clinically relevant antibiotic-resistance phenotypes have been observed in members of the genera *Enterobacter*, *Citrobacter*, *Klebsiella* or *Kluyvera*, which comprise recognized opportunistic pathogens species (Sader *et al.* 2003; Paterson 2006; Denton 2007; Hoban *et al.* 2010). Non-*E. Coli Enterobacteriaceae* are bacteria with widespread environmental distribution, which presence can be equally expected in habitats either with or without fecal contamination. *Enterobacteriaceae* excreted via animal fecal sources and those occurring naturally are supposed to cohabit, at least in some parts of the urban water cycle (raw water–drinking water–wastewater) (Leclerc *et al.* 2001). For instance, strains of the genera *Citrobacter*, *Enterobacter* or *Klebsiella* are able to proliferate in sewage, becoming predominant members of the culturable microbiota in these environments. Simultaneously, these bacteria are representatives of the drinking water bacterial flora (Brenner 1992; Leclerc *et al.* 2001; Blanch *et al.* 2007; Kampfer *et al.* 2008). Allegedly, ubiquitous *Enterobacteriaceae* can move freely between different compartments of the urban water cycle, for example from sewage and wastewater to surface water or, somehow, gain access into drinking water distribution systems. Although it is plausible to admit that such paths of propagation make these *Enterobacteriaceae* relevant vehicles for antimicrobial resistance dissemination, to our knowledge, this issue has never been addressed in literature. In a study which aims at investigating the paths and extent of antimicrobial resistance dissemination in the environment, it was intended to assess the role of non-*E. Coli Enterobacteriaceae*. With this objective, *Enterobacteriaceae* isolated from: (i) wastewaters of municipal wastewater treatment plant, (ii) urban water streams with point sources discharges, and (iii) drinking water, were characterized. The hypothesis of this study was that ubiquitous *Enterobacteriaceae*, which can colonize different types of water, could represent a vehicle of resistance dissemination. The aim of the current study was, thus, to compare the three types of water in terms of: (i) *Enterobacteriaceae* genera distribution, (ii) occurrence of resistance to 12 antibiotics, and (iii) occurrence and characteristics of the variable regions of class 1 and 2 integrons.

METHODS

Sample collection

Water samples (1 L) were collected over the period of January 2004 to November 2006 in the region of Porto in northern Portugal. The bacterial strains examined in this study were recovered through different sampling dates – eight for wastewater (WW), four for water streams (WS) and 15 for drinking water (DW). Samples of raw (RWW) and treated (TWW) wastewater were collected from a municipal treatment facility serving 85,000 inhabitant equivalents (Ferreira da Silva *et al.* 2006; Novo & Manaia 2010). Surface water samples were collected from urban water streams near the bank at a depth of 10–50 cm. Some of these streams are supposed to receive inadvertent and illegal sewage discharge by houses not connected to the municipal sewage collector. Both types of water were positive for the presence of *E. coli* (>1 CFU/100 mL), (Ferreira da Silva *et al.* 2007; Figueira *et al.* 2011). Drinking water samples, in which *E. coli* was below the allowed value (<0 CFU/100 mL), comprised a drinking water distribution system with 33 independent sampling sites (Faria *et al.* 2009) and 10 private wells (these are natural waters not submitted to any disinfection process).

Bacterial enumeration and isolation

For drinking water and water streams samples, volumes of 100 mL or 1/10/100 mL, respectively, were examined for

the presence of fecal contamination, according to the international standard method (*International Standard* ISO 9308-1 2000). The isolates included in this study correspond to samples conform to the legal recommendations for drinking water quality (Council directive 98/83/EC 1998; DL306-2007 2007). Drinking water and water streams strains were isolated on tergitol-7 agar (10.0 g/L peptone, 6.0 g/L yeast extract, 5.0 g/L meat extract, 20.0 g/L lactose, 0.05 g/L bromothymol blue, 0.1 g/L tergitol-7, 13.0 g/L agar, Oxoid; supplemented with triphenyltetrazolium chloride 0.125%, Oxoid) at 36 ± 2 °C. Wastewater strains were isolated on m-endo-agar-LES (1.2 g/L yeast extract, 3.7 g/L casitone, 3.7 g/L thiopeptone, 7.5 g/L tryptose, 9.4 g/L lactose, 3.3 g/L dipotassium phosphate, 1.0 g/L monopotassium phosphate, 3.7 g/L sodium chloride, 0.1 g/L sodium desoxycholate, 0.05 g/L sodium lauryl sulphate, 1.6 g/L sodium sulphite, 0.8 g/L basic fuchsin, 15.0 g/L agar, Difco) at 35 °C and Plate Count Agar (5.0 g/L tryptone, 1.0 g/L glucose, 2.5 g/L yeast extract and 15.0 g/L agar; Pronadisa) at 30 °C for 24 h (Ferreira da Silva *et al*. 2006). After purification, presumable *Enterobacteriaceae* were cryopreserved at −80 °C in nutrient broth with 15% (v/v) of glycerol for further identification and characterization. A total of 267 isolates of ubiquitous *Enterobacteriaceae*, 123 from drinking water (102 from a drinking water network and 21 from water wells), 33 from water streams, and 111 from wastewater (42 from RWW and 69 from TWW) were analysed in this comparative study.

Bacterial identification

All isolates were identified according to the protocol described by Ferreira da Silva *et al*. (2007). Briefly, after a preliminary characterization (Gram-staining, catalase and oxidase tests) bacteria were genotyped, using random amplified polymorphic DNA (RAPD), with primers M13 (5′-GAG GGT GGC GGT TCT-3′) and T3B (5′-AGG TCG CGG GTT CGA ATC C-3′). This procedure led to the organization of the isolates into resemblance groups. About 25% of the representatives of each resemblance group and all ungrouped isolates were identified through the analysis of 16S rRNA gene sequence (800–1,200 bp), as described before (Ferreira da Silva *et al*. 2007). As an additional confirmation, members of different groups were also identified using the API 20E system (bioMérieux). In total, the analysis of the 16S rRNA gene sequence was applied to 65% and the API 20E system to 10% of the isolates.

Antibiotic-resistance phenotypes

The antibiotic-susceptibility phenotypes were determined according to the standard disc diffusion method, as recommended by the Clinical and Laboratory Standards Institute (CLSI 2005). Twelve antibiotics were tested: amoxicillin (AML, 25 μg); ticarcillin (TIC, 75 μg); cephalothin (CP, 30 μg); ceftazidime (CEF, 30 μg); meropenem (MER, 10 μg); colistin sulphate (CT, 50 μg); sulphamethoxazole (SUL, 25 μg); sulphamethoxazole/trimethoprim (SXT, 23.75/1.25 μg); ciprofloxacin (CIP, 5 μg); tetracycline (TET, 30 μg); gentamicin (GEN, 10 μg) and streptomycin (STR, 10 μg). The reference strains *E. Coli* ATCC 25922 and *Pseudomonas aeruginosa* DSM 1117 (=ATCC 27853) were included in each experimental set as quality controls.

Antibiotic-resistance prevalence values were compared among the groups of isolates from the different types of water, using the chi-squared test (SPSS, version, 17.0 for Microsoft Windows). A significance value of $p < 0.05$ was used.

Detection and characterization of the variable regions of class 1 and 2 integrons

Detection of class 1 and class 2 integrons was performed by PCR using crude-cell lysates. The variable region of class 1 integrons was screened using primers to target the conserved sequences CS3–CS5, flanking the integron variable regions, as described before (Lévesque *et al*. 1995; Ferreira da Silva *et al*. 2007). In isolates yielding CS3–CS5 amplicons, the presence of the integrase gene was used as a confirmation of the class 1 integron presence (Henriques *et al*. 2006). Mapping analyses and sequencing of CS3–CS5 flanked regions involved the primers CS5 and aadA1 rv (ant(3″)-Ia in Lévesque *et al*. 1995), aadA1 fw (ant(3″)-I-3′ in Lévesque *et al*. 1995) and dhfrI (Lévesque *et al*. 1995; Ferreira da Silva *et al*. 2007). Class 2 integrons were screened using primers targeting the integrase and *aad*A1 genes, as described before (Laroche *et al*. 2009). The primers aadA1 fw/TnsE-Tn7 and aadA1 rv/IntI2 fw (Skurnik *et al*. 2005;

Henriques *et al.* 2006) were used to detect both the presence of a class 2 integron and screen the gene cassettes contained within the variable region. For sequencing analysis, PCR products of the variable regions of the integrons were cloned and the cloned DNA was extracted, amplified and sequenced. The commercial cloning system pGEM®-T easy vector (pGEM®-T Easy Vector System, Promega) was used according to the manufacturer instructions.

RESULTS

Distribution of genera and species

The isolates examined in this study corresponded to the most abundant culturable non-*E. Coli Enterobacteriaceae* present in each type of water. On average, the bacterial counts corresponded to 1 CFU/mL in drinking water, 10^1–10^2 CFU/mL in water streams samples, 10^3 CFU/mL in treated wastewater and 10^5 CFU/mL in raw wastewater. The genera *Citrobacter*, *Enterobacter* and *Klebsiella* were observed in the three types of water, and *Kluyvera* and *Raoultella* were observed both in drinking water and wastewater, but not in water streams (Figure 1). In drinking water, members of the genera *Klebsiella*, *Enterobacter* and *Raoultella* were predominant (24.4, 21.1 and 22.0%, respectively). In water streams, most of the isolates belonged to the genera *Klebsiella* and *Citrobacter* (57.6 and 24.2%, respectively) and in wastewater most of the isolates were members of the genera *Citrobacter, Kluyvera, Raoultella* and *Klebsiella* (21.6, 21.6, 18.0 and 15.3%, respectively). Different species of the genera *Klebsiella* and *Raoultella* prevailed in each type of water (Table 1). The species *K. oxytoca* prevailed in drinking water, *K. pneumoniae* in water streams and approximate proportions of *K. oxytoca* and *K. pneumoniae* were observed in wastewater. The species *R. planticola* and *R. terrigena* were predominant in drinking water, whereas *R. terrigena and R. ornithinolytica* prevailed in wastewater (Table 1).

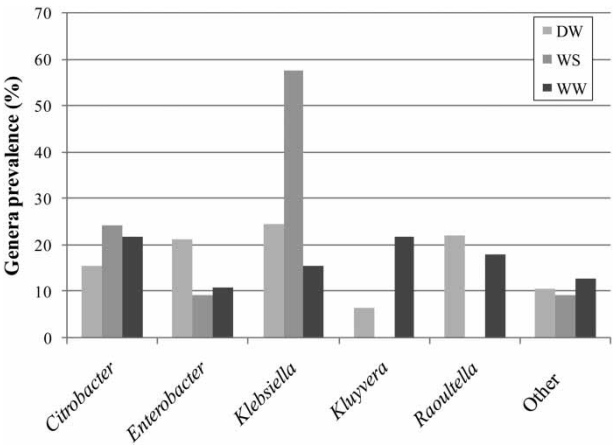

Figure 1 | Distribution of genera isolated from drinking water (DW, light bars), water streams (WS, gray bars) and wastewater (WW, dark bars) isolates examined in this study. Others include: *Erwinia carotovora* (1); *Serratia fonticola* (1); suspected new species (3); *Buttiauxella agrestis* (4); *Pantoea agglomerans* (3) and *Hafnia alvei* (1), in DW; *Pantoea agglomerans* (3) in WS; *Buttiauxella agrestis* (2); *Pantoea agglomerans* (2); *Providencia rettgeri* (1); *Providencia vermicola* (1); *Serratia* spp. (1); *Serratia marcescens* (2), *Serratia fonticola* (1); *Serratia liquefaciens* (1); *Erwinia soli* (1); *Leclercia adecarboxylata* (1) and *Morganella morganii* (1), in WW.

Antibiotic-resistance patterns

One of the objectives of this study was to compare the prevalence of antibiotic resistance in different types of water. Irrespective of the type of water, most of the isolates were susceptible to more recent antibiotics and/or those with lower consumption rates. Resistance against the antibiotics ceftazidime and meropenem was not observed in any type of water and against the antibiotics colistin sulphate, gentamicin, ciprofloxacin, tetracycline and sulphamethoxazole/trimethoprim low prevalence values were observed (Table 1). The most prevalent resistance phenotypes were observed for amoxicillin, ticarcillin, cephalothin and sulphamethoxazole. Among these, sulphamethoxazole resistance was observed to be present in every genera, with prevalence values above 25%. Resistance against the beta-lactams amoxicillin and ticarcillin presented the highest prevalence values among members of the genera *Klebsiella* and *Raoultella*. Cephalothin resistance was most prevalent among the genus *Enterobacter*, followed by *Citrobacter*.

Whenever a representative number of isolates of each genus or species was available, antibiotic-resistance prevalence values were compared for drinking water, water streams and wastewater. Significant differences were observed for amoxicillin, cephalothin and sulphamethoxazole (Table 1). These differences could be associated with a particular taxonomic group. For the antibiotic amoxicillin, with significantly higher resistance rates in water

Table 1 | Prevalence (%) of the different bacterial groups in drinking water (DW), surface water (WS) and wastewater (WW), and respective percentages of antibiotic resistance phenotypes

			% of antimicrobial resistant isolates (number of isolates)									
Bacterial group	**Prevalence (%)**	**Origin (number)**	**AML**	**TIC**	**CP**	**CT**	**SUL**	**SXT**	**CIP**	**TET**	**GEN**	**STR**
Citrobacter freundii		DW (14)	14 (2)		50 (7)		29 (4)					7 (1)
		WS (8)	25 (2)	25 (2)	13 (1)					13 (1)		13 (1)
		WW (23)	17 (4)	13 (3)	43 (10)		57 (13)			4 (1)		17 (4)
Citrobacter spp.		DW (5)			20 (1)		40 (2)	40 (2)	20 (1)	40 (2)		40 (2)
		WS (0)										
		WW (1)	100 (1)		100 (1)							
Enterobacter spp.		DW (26)	46 (12)	12 (3)	85 (22)	4 (1)	23 (6)	4 (1)		4 (1)		8 (2)
		WS (3)	33 (1)		67 (2)		33 (1)					
		WW (12)	17 (2)	8 (1)	50 (6)		25 (3)			8 (1)		8 (1)
Klebsiella oxytoca		DW (22)	68 (15)	50 (11)	9 (2)		23 (5)					14 (3)
		WS (5)	20 (1)	20 (1)	20 (1)		20 (1)		20 (1)			
		WW (9)	89 (8)	11 (1)			78 (7)					11 (1)
Klebsiella pneumoniae		DW (8)	50 (4)	50 (4)	25 (2)		63 (5)					
		WS (14)	93 (13)	50 (7)	7 (1)		50 (7)		7 (1)		7 (1)	14 (2)
		WW (8)	63 (5)	63 (5)			63 (5)					
Kluyvera ascorbata		DW (3)	100 (3)	67 (2)	33 (1)		67 (2)					
		WS (0)										
		WW (12)	33 (4)	50 (6)	25 (3)		100 (12)			17 (2)		8 (1)
Kluyvera intermadia		DW (4)	25 (1)	50 (2)	25 (1)		75 (3)					
		WS (0)										
		WW (9)	33 (3)	11 (1)	11 (1)		67 (6)		11 (1)	11 (1)		11 (1)
Kluyvera spp.		DW (1)			100 (1)		100 (1)					
		WS (0)										
		WW (3)		67 (2)			33 (1)					
Raoultella ornithinolytica		DW (4)	100 (4)	50 (2)			25 (1)					50 (2)
		WS (0)										
		WW (8)	100 (8)	88 (7)			38 (3)					
Raoultella planticola		DW (13)	69 (9)	92 (12)			31 (4)	8 (1)				8 (1)
		WS (0)										
		WW (2)	100 (2)	100 (2)			50 (1)					
Raoultella terrigena		DW (10)	10 (1)	30 (3)			60 (6)					
		WS (0)										
		WW (10)	30 (3)	40 (4)	10 (1)		40 (4)					10 (1)
Other		DW (13)	31 (4)	31 (4)	38 (5)		85 (11)					8 (1)
		WS (3)	33 (1)		100 (3)							
		WW (14)	36 (5)	7 (1)	36 (5)	21 (3)	71 (10)			7 (1)		29 (4)
Overall resistance percentage	50 25 0	DW (123)	44.7	35.0	34.1	0.8	40.7	3.3	0.8	2.4	0	9.8
		WS (33)	54.5	30.3	24.2	0	27.3	0	6.1	3.0	3.0	9.1
		WW (111)	39.6	29.7	24.3	2.7	58.6	0	0.9	5.4	0	11.7

Underlined, significantly different prevalence values.

AML, amoxicillin; TIC, ticarcillin; CP, cephalothin; CT, colistin; SUL, sulphamethoxazole; SXT, sulphamethoxazole/trimethoprim; CIP, ciprofloxacin; TET, tetracycline; GEN, gentamicin; STR, streptomycin.

No resistance against ceftazidime or meropenem was observed.

- spp. includes: *Citrobacter gillenii* and non-identified *Citrobacter* species; *Enterobacter cloacae*, *E. asburiae*, *E. aerogenes, E. hormaechei* and non-identified *Enterobacter* species; *Kluyvera cryocrescens* and non-identified *Kluyvera* species.
- Others include: *Erwinia carotovora* (1); *Serratia fonticola* (1); suspected new species (3); *Buttiauxella agrestis* (4); *Pantoea agglomerans* (3) and *Hafnia alvei* (1), in DW; *Pantoea agglomerans* (3) in WS; *Buttiauxella agrestis* (2); *Pantoea agglomerans* (2); *Providencia rettgeri* (1); *Providencia vermicola* (1); *Serratia* spp. (1); *Serratia marcescens* (2), *Serratia fonticola* (1); *Serratia liquefaciens* (1); *Erwinia soli* (1); *Leclercia adecarboxylata* (1) and *Morganella morganii* (1), in WW.

streams than in drinking water or wastewater, this was due to the high prevalence of members of the species *K. pneumoniae* in that type of water. In the same way, the higher percentage of cephalothin resistance in drinking water than in the other types of water, was mainly due to the abundance of *Enterobacter* in that type of water. Nevertheless, while in drinking water, 85% of the *Enterobacter* spp. isolates were resistant to cephalothin, in wastewater only 50% of the *Enterobacter* isolates had such a phenotype. Such a difference was not observed in *Citrobacter* spp. isolated from drinking water or wastewater, as both populations showed similar cephalothin resistance rates (42 and 46%, respectively). Sulphamethoxazole resistance rates could be ranked as water streams < drinking water < wastewater, with significant differences between these groups. Members of the genera *Citrobacter*, *Raoultella*, *Klebsiella*, and *Kluyvera*, prevailing in drinking water and/or in wastewater, were observed to be the most important harbors of sulphamethoxazole resistance in these waters.

Another aim of this study was to compare the patterns of antibiotic resistance in bacteria isolated from different types of water. In order to accomplish this objective, the possible associations of resistance phenotypes in the different types of water were assessed (Table 2). This analysis consisted of the comparison of resistance rates to one antibiotic among the resistant and susceptible bacteria to another antibiotic. Such comparisons were restricted to antibiotic-resistance phenotypes observed in a representative number of isolates and only pairs of antibiotics in which significant associations were detected are presented in Table 2. Three significant associations were detected. Resistance to the penicillins, amoxicillin and ticarcillin, was associated in the three types of water, suggesting common resistance mechanisms. In contrast, in drinking water and in wastewater, a negative association between the penicillin ticarcillin and the cephalosporin cephalothin was observed. In other words, significantly lower rates of cephalothin resistance were observed among the ticarcillin resistant than among the ticarcillin susceptible bacteria (and vice-versa). The third association was observed between streptomycin and tetracycline, in drinking water and water streams, but not in wastewater. It is noteworthy that among the 111 drinking water isolates susceptible to streptomycin none of these was resistant to tetracycline, whereas three out of the 12 resistant bacteria were tetracycline resistant.

Table 2 | Patterns of association between antibiotic-resistance phenotypes in isolates of drinking water (DW), water streams (WS) and wastewater (WW)

Antibiotic	Water type	R/S (n)	AML	TIC	CP	STR	TET
AML	DW	R (55)	100	51	31	13	2
		S (68)	0	22	37	7	3
	WS	R (18)	100	56	17	17	6
		S (15)	0	0	33	0	0
	WW	R (44)	100	59	23	11	7
		S (67)	0	6	24	10	4
TIC	DW	R (43)	65	100	9	9	0
		S (80)	34	0	48	10	4
	WS	R (10)	100	100	10	20	10
		S (23)	35	0	30	4	0
	WW	R (30)	87	100	10	13	10
		S (81)	22	0	28	10	4
CP	DW	R (42)	40	10	100	12	5
		S (81)	46	47	0	9	1
	WS	R (8)	38	13	100	13	0
		S (25)	60	36	0	8	4
	WW	R (26)	38	12	100	19	0
		S (85)	34	28	0	8	7
STR	DW	R (12)	58	33	42	100	25
		S (111)	43	35	33	0	0
	WS	R (3)	100	67	33	100	33
		S (30)	50	27	23	0	0
	WW	R (13)	38	31	38	100	8
		S (98)	40	27	20	0	5

Significant differences of antibiotic resistance prevalence between resistant (R) and susceptible (S) are indicated in shadowed cells.

Distribution and characteristics of class 1 and class 2 integrons

Class 1 integrons presented similar prevalence in drinking water and wastewater (1.6% in both types of water) and were not detected among the water streams isolates (Table 3). Class 2 integrons were less prevalent in drinking water than in the other types of water (1.6% in DW, 5.4% in WW, 6.1% in WS). In drinking water, two, out of the four integrons, were found in *K. oxytoca*, whereas in wastewater were mainly detected in *Kluyvera intermedia* (5 out of 7). Among the four class 1 integrons analysed, three, detected in isolates of wastewater and drinking water, had the *aad*A1 gene and another one, detected in a *Citrobacter gillenii* isolated from drinking water, contained the cassette

Table 3 | Class 1 and class 2 integrons: isolates origin, size, gene cassette and resistance phenotype

	Identification	Class	Size and gene cassette	Resistance phenotype
DW	*Citrobacter gillenii*	1	1.9 kb (*dfr*A12, *aad*A2)	CIP, SXT, TET, CP, SUL, STR
	Klebsiella oxytoca	2	1.5 kb (*sat2*, *aad*A1)	AML, TIC, STR
	Klebsiella oxytoca	2	1.5 kb (*sat2*, *aad*A1)	AML, TIC, SUL, STR
	Kluyvera intermedia	1	1.0 kb (*aad*A1)	TIC, SUL, STR[a]
WS	*Citrobacter freundii*	2	2.0 kb (*dfr*A1, *sat2*, *aad*A1)	AML, TET, TIC, STR
	Klebsiella pneumoniae	2	2.0 kb (*dfr*A1, *sat2*, *aad*A1)	AML, GEN, CIP, CP, TIC, SUL, STR
WW	*Klebsiella oxytoca*	1	1.0 kb (*aad*A1)	AML, SUL, STR[a]
		2	1.5 kb (*sat2*, *aad*A1)	
	Kluyvera intermedia	2	1.5 kb (*sat2*, *aad*A1)	CIP, STR
	Kluyvera intermedia	2	2.0 kb (*dfr*A1, *sat2*, *aad*A1)	TET, SUL, STR[a]
	Kluyvera intermedia	2	2.0 kb (unidentified, £)	SUL
	Kluyvera intermedia	2	2.0 kb (unidentified, £)	SUL
	Kluyvera intermedia	2	1.5 kb (*sat2*, *aad*A1)	AML, TIC, SUL
	Erwinia soli	1	1.0 kb (*aad*A1)	SUL, STR

[a]intermediate phenotype.
£, highest nucleotide similarity with a hypothetical protein of *Klebsiella pneumoniae* (acc. n. AP006725.1).

*dfr*A12, *aad*A2. This strain was resistant to sulphamethoxazole/trimethoprim, presumably due to the presence and expression of the *dfr*A12 gene. The gene cassette *sat2*/*aad*A1 was detected in eight out of the 10 class 2 integrons analysed, distributed by the three types of water. The gene *dfr*A1, encoding for trimethoprim resistance, was detected in class 2 integrons of the water streams isolates and in a *K. intermedia* isolated from wastewater.

DISCUSSION

Ubiquitous and environmental *Enterobacteriaceae* comprise, respectively, bacteria that can be found in feces and which can be easily dispersed by air and water, or psychotrophic bacteria that can proliferate in pristine or polluted waters (Leclerc *et al.* 2001). The isolates identified in this study could be allocated to both categories, emphasizing the capacity of *Enterobacteriaceae* to colonize different types of water. Different species were observed to prevail in each type of water, probably due to the sources of these bacteria and/or to the properties of the water (organic load, disinfection, etc.). Nevertheless, most of the species detected in wastewater were also found in drinking water. The presence of coliforms in drinking water has been regarded as an important marker of water safety (Leclerc *et al.* 2001). Even though, coliforms are reported in drinking water worldwide. For instance, as in the current study, Blanch *et al.* (2007) reported the occurrence of *K. oxytoca* as one of the predominant coliforms in a drinking water distribution system in Spain. Other isolates identified by those authors were members of the genera *Enterobacter*, *Citrobacter* or *Pantoea*, also identified in the current work. Kampfer *et al.* (2008), analyzing drinking water reservoirs in Germany, observed the predominance of members of the genera *Enterobacter* and *Serratia*, and low percentages of isolates of the groups *Klebsiella* and *Citrobacter*. Also these genera were identified in the present study.

Waters with fecal contamination, particularly human-derived wastewater, have been regarded as relevant reservoirs for antibiotic resistance in the environment (Baquero *et al.* 2008; Martinez 2009; Kümmerer 2009a). In contrast, drinking water, pumped in clean areas and/or disinfected (Council directive 98/83/EC 1998; DL306-2007 2007) is supposed to have low numbers of microorganisms and, thus, of antibiotic-resistant bacteria. However, the observation of the same species in waste-, surface and drinking water raised the hypothesis that coliforms can serve as a vehicle for antibiotic-resistance propagation.

This hypothesis led to the analysis and comparison of the antibiotic resistance phenotypes. The *Enterobacteriaceae* examined in this study exhibited mainly resistance against antibiotics belonging to the classes with higher consumption

rates in outpatients (amoxicillin, ticarcillin, sulphamethoxazole and cephalothin) (ESAC 2008). Other resistance phenotypes (colistin sulphate, gentamicin, ciprofloxacin, tetracycline and sulphamethoxazole/trimethoprim) were rare, irrespective of the type of water. Among these, some of the cases of colistin resistance phenotypes, in *Proteus* spp. and *Serratia* spp., are probably intrinsic (Denton 2007).

Among the most common resistance phenotypes were also some presumable situations of intrinsic resistance. For instance, members of the genera *Klebsiella* and *Raoultella* are described as intrinsically resistant to beta-lactam antibiotics (Stock & Wiedemann 2001), explaining the high rates of amoxicillin and ticarcillin resistance observed. Another situation is related to cephalothin resistance, significantly more prevalent in drinking water than in waste- or surface water, mainly due to the predominance of *Enterobacter* spp. and *Citrobacter* spp. in these types of water. This resistance phenotype is considered to be intrinsic in clinical isolates of the genera *Citrobacter* and *Enterobacter* (CLSI 2005). However, for *Enterobacter* spp. cephalothin resistance rates were lower in wastewater than in drinking water. Such results lead to the hypothesis that cephalosporin resistance favors the tolerance to the water disinfection processes. In addition, it suggests that environmental *Enterobacter* spp. may still be considered not intrinsically resistant to first generation cephalosporins.

Except for sulphonamides, the antibiotic-resistance rates in drinking water were not significantly lower than in wastewater, where fecal contamination is supposed to supply antibiotic-resistant bacteria (Kümmerer 2009a). Sulphonamides have a widespread distribution in surface and wastewaters and it is possible that these micro-pollutants impose selective pressures, mainly in wastewaters (Kümmerer 2009a, b). According to the data obtained, wastewater treatment had a limited capacity to remove sulphonamides resistance, as it reached the highest resistance rate in the treated effluent (62%, data not shown). The high rates of sulphonamides resistance prevalence in drinking water suggest that this resistance phenotype can spread easily throughout the urban water cycle.

Different patterns of association were observed in each type of water, suggesting that different sources of colonization or paths of resistance acquisition may be involved. In waste- and drinking water, cephalothin resistance was more frequent among ticarcillin susceptible isolates than among those resistant to that penicillin, hinting the involvement of distinct resistance mechanisms. In drinking water and water streams, tetracycline and streptomycin resistance were associated, suggesting that the combination of both phenotypes may improve the bacterial fitness in the environment, namely in drinking water. These patterns of association may hint the complex network of factors that rule the ecology of antibiotic resistance (Andersson & Hughes 2010).

Supposedly, bacteria thriving in habitats rich in antibiotic-resistant bacteria and/or antimicrobial residues, capable of imposing selective pressures, represent important vehicles for antibiotic-resistance dissemination (Baquero *et al.* 2008; Martinez 2009; Kümmerer 2009a, b). In this respect, the most important drivers for antibiotic-resistance propagation include genetic elements associated with processes of horizontal gene transfer. These genetic elements contain the components for site-specific recombination, which facilitate the capture and expression of mobile gene cassettes (Hall & Collis 1995). Thus, although not being mobile elements, integrons are frequently associated with antibiotic resistance acquisition (Partridge *et al.* 2009). For this reason, these genetic elements are regarded as important markers to assess the capacity of a bacterium to acquire antibiotic-resistance genes through horizontal gene transfer processes (Partridge *et al.* 2009). One of the objectives of the current work was to assess if class 1 and class 2 integrons were more prevalent and/or if different gene cassettes were detected in wastewater than in the other types of water. Although class 1 integrons presented similar prevalence values in the three types of water, class 2 integrons were more frequent in wastewater than in drinking water. Nevertheless, both types of integrons were detected in drinking water, and with similar gene cassettes. The composition of the integrons in *Enterobacteriaceae* from waste- and surface water seems to be very stable. In fact, similar gene cassettes have been reported worldwide (Tennstedt *et al.* 2003; Henriques *et al.* 2006; Ferreira da Silva *et al.* 2007; Laroche *et al.* 2009). As observed by other authors, these gene cassettes hardly can explain the observed antibiotic-resistance phenotypes (Henriques *et al.* 2006; Ferreira da Silva *et al.* 2007; Laroche *et al.* 2009). Nevertheless, integrons are regarded as gene capture systems, which under

appropriate conditions, may represent a selective advantage for antibiotic-resistance acquisition. In this respect, it is relevant to show that drinking water *Enterobacteriaceae* are equipped with the same gene capture structures that are found in waste- and surface water or in clinical isolates.

The three types of water examined in this study were sampled from areas which are not inter-connected, so a direct contamination is excluded. In particular, the area for water pumping, treatment and production of drinking water is situated several kilometres upstream from points of discharge of wastewater treatment plants. Given the ubiquity and ability that bacteria have to survive and spread in the environment, a remote contamination of drinking water from waters with fecal contamination cannot be totally discarded. The observation of antibiotic-resistant bacteria in drinking water can be explained either due to the entrance of antibiotic-resistant bacteria in some part of the water distribution system or to other unknown mechanisms of resistance emergence and/or selection, within the system. Further studies based on molecular epidemiology of these bacteria may contribute to elucidate these aspects. Altogether the data presented in this work shows that *Enterobacteriaceae*, given their ubiquity and plasticity, are important vehicles of antibiotic resistance, which under favorable conditions may colonize drinking water.

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