Antimicrobial resistance patterns in *Enterobacteriaceae* isolated from an urban wastewater treatment plant

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Enterobacteriaceae; antibiotic resistance; disinfectants; heavy metals; class 1 integron.

Abstract

Over 18 months, enterobacteria were isolated from the raw (189 isolates) and treated (156 isolates) wastewater of a municipal treatment plant. The isolates were identified as members of the genera *Escherichia* (76%), *Shigella* (7%), *Klebsiella* (12%) and *Acinetobacter* (4%). Antimicrobial susceptibility phenotypes were determined using the agar diffusion method for the antibiotics amoxicillin, gentamicin, ciprofloxacin, sulfamethoxazole/trimethoprim, tetracycline and cephalothin, the disinfectants hydrogen peroxide, sodium hypochlorite, quaternary ammonium/formaldehyde and iodine, and the heavy metals nickel, cadmium, chromium, mercury and zinc. Class 1 integrons were detected by PCR amplification using the primers CS5 and CS3. Compared with the raw influent, the treated wastewater presented higher relative proportions of *Escherichia* spp. isolates resistant to ciprofloxacin and cephalothin ($P < 0.0001$ and $P < 0.05$, respectively). Except for mercury, which showed a positive correlation with tetracycline and sulfamethoxazole/trimethoprim, no significant positive correlations were observed between antibiotic, disinfectant and heavy metal resistance. The variable regions of class 1 integrons, detected in *c*. 10% of the *Escherichia* spp. isolates, contained predominantly the gene cassettes *aadA*1/*dhfr*I.

Introduction

Contemporary populations of enteric bacteria, when compared with those from the preantibiotic era, display a higher tolerance in their nonspecific responses to several antibiotics (Houndt & Ochman, 2000). The increase in antimicrobial resistance, observed in a bacterial population, may result from the clonal selection of organisms that tolerate sublethal antimicrobial doses and that present greater fitness under conditions of selective pressure, or from the spreading of resistance genetic determinants through horizontal gene transfer. The most plausible hypothesis is that, in the natural environment, both mechanisms are responsible for the dynamics of the bacterial population. In different environments, bacteria are expected to experience distinct selective pressures for antibiotic resistance and, hence, distinct patterns of antibiotic resistance acquisition and evolution. Urban wastewater treatment plants represent important reservoirs of human and animal commensal bacteria in which antibiotic resistance determinants and/or organisms persist in the final effluent and are released to the environment (Reinthaler *et al*., 2003; Tennstedt *et al*., 2003). In a comparative study of three activated sludge treatment plants, Reinthaler *et al.* (2003) concluded that, although no significant increases in antibiotic resistance phenotypes were observed over the course of sewage treatment, this process may contribute to the dissemination of resistant bacteria to the environment. In addition, Tennstedt *et al.* (2003) reported the presence of antibiotic resistance determinants in self-transmissible genetic elements of bacteria residing in the activated sludge and final effluent released from a wastewater treatment plant.

In this study, the prevalence of antibiotic resistance was compared in *Escherichia* spp. isolated from raw and treated wastewater from an urban wastewater treatment plant. In order to assess the potential effect of other forms of chemical pollution, frequently found in human-managed environments, on these bacteria, the correlations between the growth inhibition caused by antibiotics, disinfectants and heavy metals were also assessed. The variable regions of class 1 integrons, frequently associated with antibiotic resistance, were screened and characterized.

Materials and methods

Wastewater treatment plant, sampling and analysis

Raw and treated wastewater samples were collected at six different times (winter 2004 and 2005, spring 2004 and 2005, summer 2004 and autumn 2004) from a wastewater treatment plant situated in northern Portugal (Ferreira da Silva *et al.*, 2006). The microbiological parameters, chemical oxygen demand (COD) and biochemical oxygen demand (BOD) of each water sample were determined according to standard methods, as described previously (Ferreira da Silva *et al.*, 2006). The heavy metal quantification was made according to standard methods (American Public Health Association, 1995).

Isolation and characterization of bacteria

Isolations were made from cultures corresponding to wastewater decimal dilution factors ranging between one and three. About 25–100% of the typical colonies of faecal coliforms and other atypical colonies formed on m-Faecal coliform agar (m-FC) agar were purified and preserved at $-80\,^{\circ}C$ in nutritive broth supplemented with 15% glycerol. Three hundred and fifty-nine isolates were recovered over the sampling period (six in winter 2004, 51 in spring 2004, 90 in summer 2004, 73 in autumn 2004, 64 in winter 2005 and 75 in spring 2005), and were genotyped through the random amplified polymorphic DNA (RAPD) method using crude cell extracts and the primer OPA3 (5′-AGTCAGCCAC-3′), as described previously (Ferreira da Silva *et al.*, 2006). The electrophoretic RAPD profiles were analysed visually and the isolates were grouped according to their resemblance pattern. As a quality control of RAPD analysis and of data interpretation, a group of four isolates randomly selected from the test strains was included in each experimental set (PCR and electrophoresis). Randomly selected isolates, representative of each RAPD group, were identified through two different methodologies, with both identification methods giving similar results. Twenty-six isolates were identified using the API 20 E biochemical test system, according to the manufacturer's instructions (BioMérieux), and 43 through the analysis of the 16S rRNA gene sequence. The nucleic acid sequence of the 16S rRNA gene was determined after PCR amplification from total DNA extracts using the primers 27f and 1492r (Lane, 1991) and the following conditions: 5 min at 95 °C, followed by 30 cycles of amplification (Thermalcycler, Biometra) of 1 min at 94 °C, 1 min at 55 °C and 1.5 min at 72 °C, and a final extension step of 10 min at 72 °C. The 16S rRNA gene was amplified by PCR in a reaction mixture of 50 μL containing 1 × PCR buffer (75 mM Tris-HCl, pH 8.8 at 25 °C, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 2.5 U of Taq polymerase (Fermentas), 1 μM of each primer and 2 μL of crude cell lysate. The PCR products were purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences), according to the manufacturer's instructions. The sequences were determined using a model ABI 3700 DNA Analyser (Applied Biosystems), and their quality was checked manually using the Bioedit editor (Hall, 1999). These sequences were compared with others deposited in the public databases GenBank and EMBL.

Determination of antibiotic, disinfectant and heavy metal resistance phenotypes

The growth inhibition caused by antibiotics, disinfectants and heavy metals was assayed using the agar diffusion method. The antibiotic resistance phenotypes were determined for all isolates, according to the procedures described previously (Ferreira da Silva *et al.*, 2006). The adopted interpretation criteria based on inhibition zone diameters were as follows (mm): amoxicillin 25: $R < 14$, $I = 14$–20, $S \geq 21$; gentamicin 10: $R \leq 14$, $S \geq 15$; ciprofloxacin 5: $R < 19$, $I = 19$–21, $S \geq 22$; tetracycline 30: $R < 17$, $I = 17$–18, $S \geq 19$; sulfamethoxazole/trimethoprim 25: $R < 10$, $I = 10$–15, $S \geq 16$; sulfamethoxazole 25: $R < 12$, $I = 12$–15, $S \geq 16$; cephalothin 30: $R \leq 14$; streptomycin 10: $R < 13$, $S \geq 15$. The strains *Escherichia coli* ATCC 25922, *Enterococcus faecalis* DSM 2570, *Pseudomonas aeruginosa* DSM 1117 and *Staphylococcus aureus* DSM 1104 were included in each experimental set as controls. The average deviation of the diameters of the inhibition zones measured for these control strains ranged between 1 and 2 mm.

A group of *Escherichia* spp. isolates was further characterized for relative susceptibility to disinfectants and heavy metals. The tested strains included approximately equal proportions of bacteria susceptible to all antibiotics and resistant at least one, and representatives of the raw and treated wastewater. The disinfectants assayed were commercial hydrogen peroxide (3%, w/w) (HP), commercial bleach (< 5% w/w sodium hypochlorite) (HC), a 25% (v/v) solution of a commercial disinfectant recommended for the disinfection of hospital facilities and equipment, and clinical laboratories (didecyldimethylammonium–fat alcohol ethoxylate–dialdehyde–formaldehyde, 10 : 5 : 5 : 5, MEDV6, Diversey Lever) (quaternary ammonium/formaldehyde, QAF) and a 3% iodine solution (1% w/v of iodine crystals, 2% w/v of potassium iodide) (IS). The heavy metal salt solutions (Merck) assayed were as follows: 50 mM $Cd(NO_3)_2 \cdot 4H_2O$, 200 mM $ZnSO_4$, 200 mM $NiCl_2 \cdot 6H_2O$, 50 mM $K_2Cr_2O_7$ and 50 mM $HgCl_2$. The bacterial suspensions and inoculations were performed as described previously (Ferreira da Silva *et al.*, 2006), using plate count agar and blank discs (Oxoid) in the case of disinfectants. Each disc was

impregnated with 10 µL of HP, HC or QAF, or 15 µL of IS. For heavy metal inhibition tests, a drop of 10 µL of cadmium, chromium and mercury solutions, or 15 µL of zinc and nickel solutions, was applied directly onto the inoculated medium. After an incubation period of 24 h at 35 °C, the diameters of growth inhibition were measured. *Escherichia coli* ATCC 25922, included in each experimental set as a control, presented a variation in inhibition diameter of 1–2 mm. The variations in the inhibition diameter (mm) obtained for antibiotics, disinfectants and heavy metals were as follows: amoxicillin, 0–28; gentamicin, 0–23; ciprofloxacin, 0–38; tetracycline, 0–32; sulfamethoxazole/trimethoprim, 0–33; sulfamethoxazole, 0–25; cephalothin, 0–26; streptomycin, 0–15; HP, 19–36; HC, 17–40; IS, 15–25; QAF, 0–40; nickel, 13–29; cadmium, 11–24; zinc, 12–22; mercury, 14–35; chromium, 26–42.

Detection and characterization of the variable regions of class 1 integrons

The screening and characterization of the variable regions of class 1 integrons followed the procedures described previously (Lévesque *et al*., 1995; Martinez-Freijo *et al*., 1998; Maguire *et al*., 2001). The same DNA extracts used for genotyping were screened for the presence of variable regions of class 1 integrons, ensuring the quality of the DNA extracts. The primers CS5 (5′-GGCATCCAAGCAGCAAG-3′) and CS3 (5′-AAGCAGACTTGACCTGA-3′) were used to detect the variable regions of class 1 integrons. The primers ant(3″)-Ia, ant(3″)-I-3′ and dhfrI were used to map the respective variable regions, as described by Lévesque *et al*. (1995). PCR amplifications (Thermalcycler, Biometra) were prepared as follows: 1 U of Taq polymerase (Fermentas), 1 × PCR buffer (10 mM Tris-HCl, pH 8.8 at 25 °C, 50 mM KCl, 0.08% Nonidet P40), 2.5 mM $MgCl_2$ (Fermentas), 0.2 mM of each dNTP (Fermentas), 1 µM of each of the forward and reverse primers and 15 µL of the crude cell lysate, in a total volume of 50 µL. The amplification programme was as follows: 10 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 54 °C and 2 min at 72 °C, and a final extension of 10 min at 72 °C. PCR products were analysed and purified by electrophoretic separation in a 1.5% agarose gel in Tris-acetate–EDTA buffer. The variable regions of the class 1 integrons were partially sequenced using the primers CS3, ant(3″)-Ia, ant(3″)-I-3′ and/or dhfrI.

Statistical analyses

The chi-squared test was used to compare the prevalence of antibiotic resistance phenotypes amongst the isolates from raw and treated wastewater, and the resistance prevalence and presence of integron-specific gene cassettes amongst the isolates resistant and susceptible to each antibiotic. The correlations between the inhibition diameters caused by antibiotics, disinfectants and heavy metals were assessed by Pearson correlation analysis. For all the statistical analyses, the application SPSS 14.0 for Windows (SPSS Inc., Chicago, IL) was used.

Results and discussion

Wastewater characterization

The chemical and microbiological parameters of raw and treated wastewater for the sampling period winter 2004 to autumn 2004 have been reported previously (Ferreira da Silva *et al*., 2006), and were included in the calculations presented in Table 1. The microbiological analysis of raw wastewater over the sampling period winter 2004 to spring 2005 showed that the numbers of total heterotrophs and total coliforms were in the range 10^7–10^8 CFU 100 mL^{-1}, whereas the numbers of faecal coliforms and enterococci were in the region of 10^6 CFU 100 mL^{-1} (Table 1). Treatment produced a decrease of 80–90% in the levels of the organisms in each of these groups, corresponding to an average $\log_{10}$ decrease of 0.8–1.3. The average decreases in COD and BOD observed in the same period were 74% and 81%, respectively, with the average COD and BOD values in treated wastewater in the legally established range (125 and 25 mg O_2 L^{-1}, respectively) (Council Directive 91/271/EEC, 1991).

Table 1. Chemical and microbiological analyses of raw and treated wastewater samples used in this study

Parameter	Raw water, median (range)	Treated water, median (range)	$\log_{10}$ reduction, average ± SD	Removal (%), average ± SD
Total heterotrophs (CFU × 10^5/100 mL)	2200 (520–4900)	220 (65–380)	1.0 ± 0.3	89 ± 5.4
Total coliforms (CFU × 10^5/100 mL)	450 (220–1400)	58 (20–260)	0.8 ± 0.4	79 ± 14.5
Faecal coliforms (CFU × 10^5/100 mL)	36 (25–53)	3 (1–6)	1.3 ± 0.4	92 ± 6.9
Faecal streptococci (CFU × 10^5/100 mL)	41 (26–85)	3 (1–5)	1.2 ± 0.4	92 ± 4.7
COD (mg O_2 L^{-1})	400 (290–720)	91 (71–230)	0.7 ± 0.3	74 ± 18.6
BOD (mg O_2 L^{-1})	250 (170–400)	23 (16–130)	0.9 ± 0.4	81 ± 20.3

BOD, biochemical oxygen demand; COD, chemical oxygen demand.

Isolation, genotyping and identification of m-FC isolates

Three hundred and fifty-nine isolates was purified from m-FC agar, a selective medium used to isolate and presumptively identify faecal coliforms (American Public Health Association, 1995). The RAPD-based genotyping of the isolates resulted in the definition of three major resemblance groups identified using the API 20 E system and/or16S rRNA gene sequence analysis. The largest RAPD group integrated 290 isolates. Within this largest cluster, it was possible to distinguish a subgroup of 26 isolates that, despite the general resemblance pattern, presented a characteristic set of very low-intensity bands. These isolates were identified, through 16S rRNA gene sequence analysis, as members of the genus *Shigella*, related to the species *S. sonnei*, *S. boydii* and *S. flexneri*. The other isolates integrated in the largest RAPD group were identified as *Escherichia coli* and only three as *Escherichia fergusonii*. Another RAPD group, composed of 43 elements, was identified (four through 16S rRNA gene sequence analysis and 10 through API 20 E) as *Klebsiella pneumoniae*. The smallest RAPD group contained 14 *Acinetobacter* spp. isolates. Two *Betaproteobacteria*, isolated from the treated wastewater in two different sampling periods, showed a 16S rRNA gene similarity below 97% with members of the family *Neisseriaceae*. These two *Betaproteobacteria* and the *Acinetobacter* spp. were not included in further data analysis.

The relative abundance of each genus revealed that, as expected, the most abundant isolates were members of the genus *Escherichia*, although some of these did not form the typical blue colonies on m-FC agar. *Shigella* spp., closely related to the genus *Escherichia*, occasionally formed blue colonies, whereas *K. pneumoniae* and *Acinetobacter* spp. formed atypical colonies. Bacteria belonging to these three genera were isolated in smaller proportions (Fig. 1). Wastewater treatment led to a slight decrease in the relative proportions of *Escherichia* spp. isolates, with a concomitant increase in *Acinetobacter*, *Shigella* and *Klebsiella*.

Effect of wastewater treatment on antibiotic resistance phenotype

The *Enterobacteriaceae* were characterized for their antibiotic resistance phenotype (Table 2). *Klebsiella pneumoniae* isolates presented a high percentage of resistance to the antibiotic amoxicillin, a result that was expected from previous indications of the natural resistance of members of the genus *Klebsiella* to this antibiotic (Stock & Wiedemann, 2001). The results obtained for *K. pneumoniae* isolates suggest that wastewater treatment is accompanied by an increase in resistance to the other antibiotics tested. With the exception of amoxicillin and cephalothin, similar results were observed for *Shigella* spp. isolates. Two treated wastewater isolates belonging to this genus were simultaneously resistant to amoxicillin, sulfamethoxazole/trimethoprim, tetracycline and ciprofloxacin or gentamicin, respectively. However, the small number of *K. pneumoniae* and *Shigella* spp. obtained in this study does not allow a more in-depth discussion of these results.

For *Escherichia* spp. isolates, the highest antibiotic resistance prevalence was observed for amoxicillin and tetracycline, with values around 30% (Table 2). A comparison of the antibiotic resistance frequency in *Escherichia* spp. isolates from raw wastewater vs. treated wastewater revealed that, for the antibiotics amoxicillin, ciprofloxacin, tetracycline and cephalothin, the resistance prevalence increased by 5–10% in treated wastewater (Table 2). The highest increases were observed for ciprofloxacin (7%, $P < 0.0001$) and cephalothin (10%, $P < 0.01$). The resistance to multiple antibiotics showed a similar pattern of variation in raw and treated wastewater, with a decrease of *c.* 10% along the

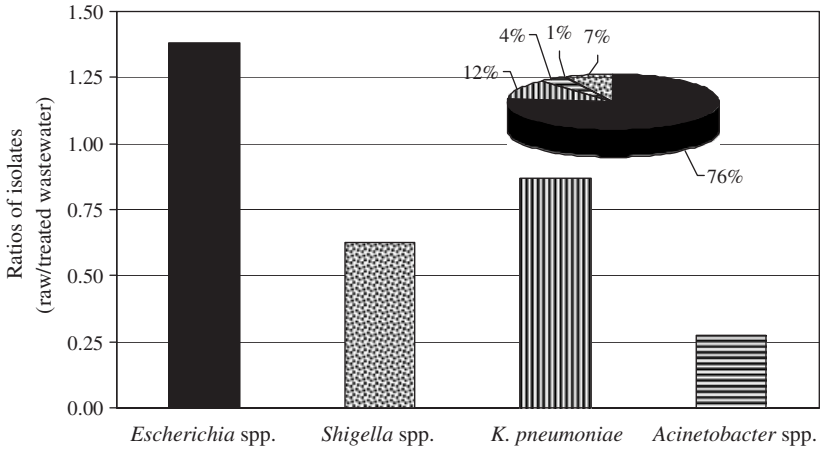

Fig. 1. Frequency of isolates belonging to different genera, recovered on the selective medium m-FC agar (pie; 1% corresponds to unidentified *Betaproteobacteria*), and the respective ratios in raw and treated wastewater.

Table 2. Prevalence (%) of antibiotic resistance (or intermediate) phenotypes in enteric bacteria isolated from raw and treated wastewater

			Resistance prevalence (%)								
Taxon	Wastewater	*n*	AML	CIP	SXT	TET	CP	GEN	R2	R3	R4
Escherichia spp.	Raw	159	28.0 (3.2)	2.5 (0.6)	22.2 (1.3)	32.1 (0.6)	10.5	3.8	28.3	16.4	5.0
	Treated	115	34.8 (6.3)	9.7 (0.9)	22.5	36.8	20.5	5.2	34.2	21.1	8.8
	Total	274	30.9 (3.8)	5.5 (0.6)	22.3 (0.5)	34.1 (0.2)	14.5	4.4	30.7	19.0	7.7
Shigella spp.	Raw	10	20.0	0.0	0.0	0.0	12.5	0.0	0.0	0.0	0.0
	Treated	16	12.5 (6.3)	6.3	12.5	25.0	0.0	6.3	12.5	12.5	12.5
	Total	26	14.8 (7.4)	3.7	7.4	14.8	4.8	3.7	7.4	7.4	7.4
Klebsiella spp.	Raw	20	94.7	0.0	0.0	5.0	0.0	0.0	5.0	0.0	0.0
	Treated	23	95.7	4.4	8.7	13.0	5.9	4.4	17.4	8.7	4.4
	Total	43	92.9	2.3	4.7	9.6	3.5	2.3	11.6	4.7	2.3

AML, amoxicillin; CIP, ciprofloxacin; CP, cephalothin; GEN, gentamicin; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline; R2, R3, R4, resistant to two or more, three or more and four or more antibiotics, respectively.

categories R2 (resistant to two or more antibiotics), R3 (resistant to three or more antibiotics) and R4 (resistant to four or more antibiotics). Each of these categories showed an increase of 3–6% after wastewater treatment (Table 2).

The results showed that wastewater treatment was accompanied by a generalized increase in antibiotic resistance and multiresistance prevalence in *Escherichia* spp. isolates. The most significant increase was observed for the antibiotic ciprofloxacin. In a previous study with enterococci from the same wastewater treatment facility, a similar effect was observed (Ferreira da Silva *et al*., 2006). The occurrence and stability of this antibiotic, as well as tetracycline and sulfamethoxazole, in the environment, namely in sewage water, have been reported previously (Halling-Sorensen *et al*., 1998; Kümmerer, 2004), and may explain these results. However, further studies are needed to provide some insights into this issue. Considering their frequent clinical use in outpatients, Stelling *et al*. (2005) have hypothesized that resistance to certain antimicrobials, such as ciprofloxacin, may be associated more with the patterns of antimicrobial use in the community rather than in hospitals.

It is assumed that commensal and environmental bacteria will present a comparatively lower antibiotic resistance prevalence than their counterparts isolated from clinical samples or from the hospital environment. Multiple surveillance programmes have provided large data sets on antimicrobial susceptibility in clinically relevant bacteria, allowing a comparison of clinical isolates worldwide (Stelling *et al*., 2005), as well as isolates from different environmental origins. Comparing the results obtained in this study with those presented in an integrating analysis of different surveillance programmes conducted worldwide for *Escherichia coli* in the year 2001 (Stelling *et al*., 2005), it can be observed that the resistance prevalence values for sulfamethoxazole/trimethoprim, ciprofloxacin and amoxicillin are of the same order of magnitude as the values arbitrarily categorized by the authors as 'low resistance' for sulfamethoxazole/trimethoprim (0–20%), ciprofloxacin (< 10%) and ampicillin (20–40%). The countries of southern Europe included in such categories were Italy for ampicillin and sulfamethoxazole/trimethoprim, and France for ciprofloxacin. The resistance prevalence of gentamicin was below 10%, in general, in the countries analysed in these surveillance programmes, a value also in accordance with that found in the present study. Nevertheless, it should be emphasized that the data in Stelling *et al*. (2005) refer to clinical isolates, which would be expected to be comparatively more resistant than bacteria from a community origin, namely from an urban wastewater treatment plant.

One of the major concerns related to the persistence of antibiotic resistance in treated wastewater is potential environmental contamination, namely of natural waters. In their study of the impact of an urban effluent on the antibiotic resistance of *Enterobacteriaceae* in a riverine area in the north of Spain, Goñi-Urriza *et al*. (2000), using a methodology similar to that employed in this study, found high resistance prevalence values for quinolones (20%), tetracycline (18%) and β-lactams (13%), with higher percentages detected downstream from the wastewater discharge. These results demonstrate that antibiotic-resistant bacteria may persist in natural waters, and may present a selective advantage relative to their susceptible counterparts in environments with different forms of chemical pollution (e.g. McArthur & Tuckfield, 2000). According to such evidence, the results obtained in this study suggest that wastewater treatment may contribute to the environmental dissemination of antibiotic-resistant bacteria.

Relative susceptibility to antibiotics, heavy metals and disinfectants

The association between antibiotic resistance and reduced susceptibility to disinfectants and heavy metals has been widely reported (Alonso *et al*., 2001). In order to assess

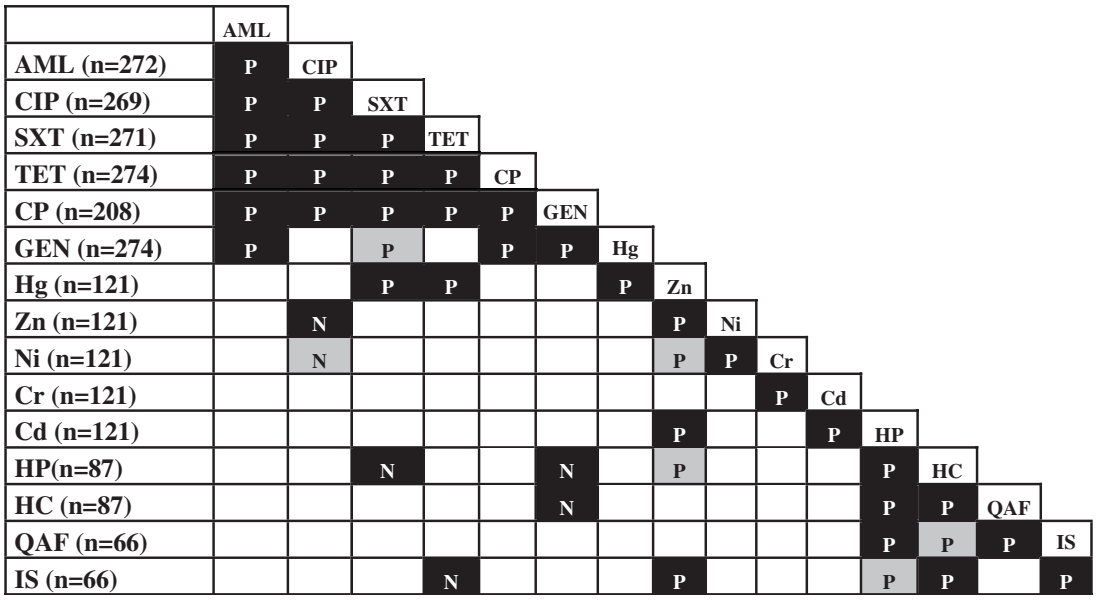

Fig. 2. Pearson correlation between the inhibition diameters produced by different antimicrobial agents. HC, sodium hypochlorite (commercial bleach); HP, hydrogen peroxide; IS, iodine solution; QAF, quaternary ammonium/formaldehyde disinfectant. Black, significant correlation at the 0.01 level; grey, significant correlation at the 0.05 level; N, negative correlation; P, positive correlation.

whether antibiotic resistance was correlated with a reduced susceptibility to disinfectants and heavy metals in these *Escherichia* spp. isolates, a group of strains was grown in the presence of four disinfectants and five heavy metals. Given the objective of this study, it was considered relevant to test household disinfectants and disinfectants similar to those used in potable water disinfection, belonging to different chemical classes. Some heavy metals frequently reported as environmental contaminants were also selected. The possible association between the patterns of inhibition produced by the antimicrobial agents, disinfectants and heavy metals was assessed through a Pearson correlation analysis (Fig. 2). Heavy metals have been widely reported as possible factors of selective pressure for antibiotic resistance, mainly as a result of the genetic linkage of resistance determinants in the same plasmid or mobile genetic element (Bass *et al.*, 1999; Alonso *et al.*, 2001). Of the heavy metals tested, a positive correlation ($r = 0.3$, $P < 0.005$) was demonstrated only between mercury and the antibiotics sulfamethoxazole/trimethoprim and tetracycline (Fig. 2). Over the sampling period, of the heavy metals included in this study, mercury presented the lowest concentrations in sludge dry matter (0.8–1.7 mg kg^{-1}), and zinc presented the highest values (1500–1700 mg kg^{-1}). In treated wastewater, mercury presented values in the range 0.5–1.2 μg L^{-1}, whereas the other heavy metals were below the detection limit (0.1 mg L^{-1}). Although mercury is present at lower concentrations than the other metals tested, it may be responsible for a selective effect. Indeed, the genetic linkage of antibiotic resistance to mercury salts has been described previously in Gram-negative bacteria (Wireman *et al.*, 1997), namely in IncP plasmids isolated from the activated sludge of a wastewater treatment plant (Dröge *et al.*, 2000). These results support the observations of McArthur & Tuckfield (2000) in stream bacteria, who reported that, in areas in which no antibiotic-resistant bacteria were discharged, a positive correlation between antibiotic resistance prevalence and mercury concentration was observed. Such evidence suggests that this metal may act as a selective factor for the proliferation of antibiotic-resistant bacteria.

The cross-resistance between antibiotics and disinfectants has been reported by some authors to be responsible for the selection of antibiotic-resistant bacteria, namely in the domestic environment (Levy, 2002; Kümmerer, 2004). However, several studies have demonstrated a lack of association between the two resistance phenotypes (e.g. Cole *et al.*, 2003). Of the disinfectants tested, none demonstrated a positive correlation with any of the antibiotics assayed. The negative correlations observed for disinfectants and antibiotics (Fig. 2) may suggest that disinfectants and/or antiseptics containing oxidative agents, such as HP or others with similar action mechanisms, may limit the proliferation and spread of sulfamethoxazole/trimethoprim- and gentamicin-resistant *Escherichia* spp.

The inhibitory effects of the disinfectants HP, HC and QAF were positively correlated in the tested isolates ($r = 0.3$–0.4, $P < 0.05$). The observation of a positive correlation between the disinfectants HP, HC and QAF, and between the first two and IS, may be explained on the basis of a similar mode of cell response to the injuries imposed by

Table 3. Prevalence of antibiotic resistance and class 1 integron variable regions amongst resistant and susceptible isolates of *Escherichia* spp

	Frequency (%)									
	AML	CIP	SXT	TET	CP	GEN	R2	R3	R4	C1 I
Amoxicillin (AML)										
Resistant ($n = 83$)	**100**	**16**	**58**	**66**	**22**	**10**	**88**	**58**	**22**	**21**
Susceptible ($n = 176$)	**0**	**1**	**6**	**21**	**5**	**1**	**6**	**1**	**0**	**6**
Ciprofloxacin (CIP)										
Resistant ($n = 14$)	**93**	**100**	**71**	**86**	29	7	**100**	**100**	**64**	14
Susceptible ($n = 257$)	**27**	**0**	**19**	**32**	10	4	**25**	**14**	**4**	10
Sulfamethoxazole/trimethoprim (SXT)										
Resistant ($n = 60$)	**80**	**17**	**100**	**78**	13	**13**	**95**	**75**	**27**	**32**
Susceptible ($n = 207$)	**16**	**2**	**0**	**21**	10	**2**	**11**	**3**	**1**	**3**
Tetracycline (TET)										
Resistant ($n = 93$)	**59**	**13**	**51**	**100**	12	**10**	**69**	**51**	**19**	**24**
Susceptible ($n = 179$)	**16**	**1**	**7**	**0**	11	**1**	**10**	**2**	**0**	**3**
Cephalothin (CP)										
Resistant ($n = 30$)	**60**	13	27	37	**100**	7	**63**	**40**	**27**	10
Susceptible ($n = 178$)	**25**	3	20	32	**0**	3	**23**	**15**	**2**	9
Gentamicin (GEN)										
Resistant ($n = 12$)	67	8	**67**	75	17	**100**	**83**	**83**	**58**	25
Susceptible ($n = 262$)	29	5	**20**	32	11	**0**	**27**	**16**	**4**	9
Class 1 integron (C1 I) variable region										
Present ($n = 27$)	**63**	7	**70**	**82**	11	11	**78**	**48**	**15**	**100**
Absent ($n = 247$)	**27**	5	**17**	**29**	11	4	**24**	**15**	**5**	**0**

R2, R3, R4, resistant to two or more, three or more or four or more antibiotics, respectively. Bold values, $P < 0.0001$; underlined values, $P < 0.005$; normal type, no significant differences; C1I, class 1 integron.
Intermediate phenotypes were excluded from this analysis.

these agents. The disinfectants HP, HC and IS act as oxidative agents (although the exact mechanisms of the last two are not fully understood), whereas QAF contains formaldehyde, which acts as a mutagenic agent interacting with nucleic acids and proteins, and a quaternary ammonium detergent, which facilitates penetration into the cell (McDonnell & Russel, 1999). Acquired bacterial resistance to disinfectants has been described, and may result from mutation or from mobile genetic elements such as plasmids or transposons (McDonnell & Russel, 1999; Gilbert & McBain, 2003). However, many bacteria are intrinsically resistant to disinfectants, which may trigger cell defensive mechanisms, such as the SOS response (McDonnell & Russel, 1999). The use of a nonspecific defensive reaction may explain the present set of results, as a clear correlation was observed between the different disinfectants, without any evident positive association with antibiotic resistance.

Patterns of resistance per group of antibiotic-resistant bacteria

Correlation analysis (Fig. 2) based on the diameters of growth inhibition demonstrated significant positive correlations between all the antibiotics tested, except gentamicin, with correlation values in the range $r = 0.2$–0.6. Gentamicin presented positive correlations with amoxicillin, cephalothin and sulfamethoxazole/trimethoprim, but not with ciprofloxacin or tetracycline. In order to better understand the positive correlations observed and to characterize the patterns of antibiotic resistance in the wastewater *Escherichia* spp. isolates, the relative frequency of antibiotic resistance was calculated for bacteria resistant and susceptible to each antibiotic (Table 3). It was observed that bacteria resistant to amoxicillin were significantly more resistant to the other antibiotics tested, significantly more multiresistant and showed a higher prevalence of class 1 integrons than did the amoxicillin-susceptible counterparts. Eighty-nine per cent of the bacteria resistant to amoxicillin were also resistant to at least one other antibiotic, mostly to tetracycline (66%) or sulfamethoxazole/trimethoprim (58%). Similar results were observed for the isolates resistant to tetracycline and sulfamethoxazole/trimethoprim. When compared with their susceptible counterparts, they were significantly more resistant to the other antibiotics tested, except for cephalothin, significantly more multiresistant and showed a higher prevalence of class 1 integrons. Multiresistance in sulfamethoxazole/trimethoprim-resistant isolates was highly prevalent (97%) and superior to that observed in tetracycline-resistant bacteria (69%). In either case, multiresistance was observed mainly with amoxicillin and tetracycline or sulfamethoxazole/trimethoprim, respectively.

Bacteria resistant to ciprofloxacin, when compared with susceptible isolates, were significantly more resistant to amoxicillin, tetracycline and sulfamethoxazole/trimethoprim and presented a higher prevalence of multiresistance. Indeed, all bacteria resistant to ciprofloxacin were resistant to another antibiotic, and more than 90% were resistant to two other antibiotics, mainly amoxicillin (93%), tetracycline (80%) or sulfamethoxazole/trimethoprim (67%). In clinical isolates of *Enterobacteriaceae*, ciprofloxacin resistance typically seems to be part of the multiresistance phenotype, occurring together with resistance to β-lactams, ceftriaxone or tetracycline (e.g. Wang *et al.*, 2001; Leverstein-van Hall *et al.*, 2003). Class 1 integrons were not more prevalent in ciprofloxacin-resistant than in ciprofloxacin-susceptible bacteria, confirming previous reports (Leverstein-van Hall *et al.*, 2003). However, the plasmid-borne quinolone resistance determinant *qnr* has been associated with both integrons and multiresistance phenotypes (Tran & Jacoby, 2002; Li, 2005; Mammeri *et al.*, 2005). The observed association between resistance to ciprofloxacin and resistance to amoxicillin, sulfamethoxazole/trimethoprim or tetracycline was consistent with the significant increase in ciprofloxacin resistance in isolates from treated wastewater. Bacteria simultaneously resistant to ciprofloxacin and to tetracycline, amoxicillin or sulfamethoxazole/trimethoprim were significantly more prevalent in treated than in raw wastewater ($P < 0.01$). These results may suggest genetic linkage between different resistance determinants, that bacteria resistant to one of these antibiotics may have higher probabilities of acquiring fluoroquinolone resistance, or that a single mechanism, for example an efflux complex (Pagès *et al.*, 2005), is responsible for more than one resistance phenotype.

Bacteria resistant to gentamicin showed a high percentage ($> 80\%$) of double and triple resistance to amoxicillin, sulfamethoxazole/trimethoprim and also tetracycline. Indeed, this antibiotic has been reported to be associated with multiresistance patterns (Leverstein-van Hall *et al.*, 2003). Cephalothin-resistant bacteria were significantly more multiresistant and presented a higher prevalence of resistance to amoxicillin, but not to the other antibiotics tested. The use of a common resistance mechanism may explain the association between amoxicillin and cephalothin resistance, for example, as a result of extended-spectrum β-lactamases (e.g. Saladin *et al.*, 2002). Although no significant increase in amoxicillin resistance accompanied wastewater treatment, bacteria simultaneously resistant to cephalothin and amoxicillin were significantly more prevalent in the treated effluent ($P < 0.005$).

The association between the resistance phenotypes to β-lactam antibiotics and those to other antibiotics of distinct chemical families, observed in this study, has been described previously for clinical and animal isolates. For example, Oteo *et al.* (2002), in a study with invasive isolates of *Escherichia coli*, observed that the prevalence of resistance to sulfamethoxazole/trimethoprim, gentamicin and ciprofloxacin was higher in ampicillin-resistant strains than in ampicillin-susceptible strains. In that study (in which tetracycline was not included), the most common type of multiresistance was ampicillin, sulfamethoxazole/trimethoprim and ciprofloxacin. In addition, in a study with *Escherichia coli* isolated from newborn calves, an association was observed between ampicillin, tetracycline and sulfamethoxazole (Hoyle *et al.*, 2005).

Variable regions of class 1 integrons

Class 1 integrons have been widely associated with antibiotic resistance acquisition and dissemination, acting mainly as resistance gene capture systems (Maguire *et al.*, 2001; Barlow *et al.*, 2004). The study of the distribution of class 1 integrons in the isolates analysed during this work was made with two major objectives. The first was to investigate whether wastewater treatment had any influence on the size and characteristics of the distribution of the variable regions of class 1 integrons. The second was to compare the class 1 integrons of the isolates analysed in this study with those of other reports focused on bacteria taxonomically similar but isolated from different origins. Thirty-one class 1 integrons, with integron-specific variable regions with sizes in the range 0.9–3 kbp, were found in the 343 enterobacteria screened (27 in *Escherichia* spp., one in *Shigella* spp., three in *K. pneumoniae*). Amongst the *Escherichia* spp., a similar frequency of class 1 integrons was found in the raw (10.0%) and treated (9.6%) wastewater. The percentage of class 1 integrons in these isolates seemed to be inferior to that reported by other authors for *Escherichia coli* from hospital, cattle and avian origins (Martinez-Freijo *et al.*, 1998; Bass *et al.*, 1999; Maguire *et al.*, 2001; Reyes *et al.*, 2003; Barlow *et al.*, 2004), but superior to that reported for an estuarine environment (Rosser & Young, 1999). In a study of antibiotic resistance plasmids isolated from a wastewater treatment plant, Tennstedt *et al.* (2003) detected integron-specific regions, with sizes in the range 1.2–3.0 kbp, in 12% of 97 multiresistance plasmids. However, not all of these authors used the same PCR primers for class 1 integron detection. Some used CS primers, whereas others used integrase-directed primers. It is not clear whether one of these methods may lead to an underestimation of these genetic elements. According to Martinez-Freijo *et al.* (1998), the 5′ CS region of class 1 integrons may present some variability, a fact that may hamper the amplification of some integron-specific gene cassettes. In addition, the absence of the 3′ CS region in class 1 integrons serving as a platform for the integration of transposable elements in IncP-1α plasmids from wastewater treatment plants has been reported

(Tennstedt *et al*., 2005). These reports show that the use of primers specific for the CS regions of class 1 integrons may lead to an underestimation of the class 1 integron-associated gene cassettes. It is known that the 3′ CS region contains a gene, *sulI*, responsible for the phenotype of sulfamethoxazole resistance (Martinez-Freijo *et al*., 1998). In the present study, only one *Escherichia* spp. isolate in which an integron-specific amplicon was detected was susceptible to sulfamethoxazole. This observation may be a result of the absence of the *sulI* gene in some class 1 integrons, as described previously (Rosser & Young, 1999). In *Escherichia* spp. isolates from raw wastewater, the predominant (13/16) integron-specific variable region size was 1.5 kbp or more, whereas, in isolates from treated wastewater, amplicons larger than 1.5 kbp were found in only six of the 11 integron-positive strains. The other five amplicons detected in treated wastewater isolates contained 1 kbp. This observation finds support in the study of estuarine isolates by Rosser & Young (1999), who described a decrease in the integron-specific variable region size as a result of dilution by the chemical agents responsible for the selective pressure effect.

Sequence analysis of the integron-specific regions, as well as mapping produced using different combinations of the primers CS3, CS5, ant(3″)-Ia, ant(3″)-Ia-3′ and dhfrI (Lévesque *et al*., 1995), revealed that, in the majority of isolates, the integron-specific region contained an aminoglycoside-adenyltransferase gene (*aadA*), frequently associated with a dihydrofolate reductase gene (*dhfr*), which mediate resistance to aminoglycosides and trimethoprim, respectively. Of the *aadA* genes, *aadA*1 was detected in the majority (20/27) of the *Escherichia* spp. strains and in the unique *Shigella* integron (1 kbp) detected. This gene was associated (except in one isolate) with the phenotypic expression of streptomycin resistance. The *aadA*5 gene was observed in three *Escherichia* spp. isolates, which exhibited susceptibility to gentamicin and resistance to streptomycin. The aminoglycoside-2″-adenylyltransferase gene (*aadB*) was detected in a single integron gene cassette of a gentamicin- and streptomycin-resistant *Escherichia* spp. isolate. The streptothricin acetyltransferase gene (*sat*1) was detected in a single isolate susceptible to gentamicin and resistant to streptomycin. When present (14 *Escherichia* spp. isolates), the dihydrofolate reductase gene *dhfr*I was found upstream of the *aadA*1 gene, and was associated with resistance to sulfamethoxazole/trimethoprim. The observed associations between the genes contained in the integrons and the resistance phenotypes were in agreement with previous descriptions (Rosser & Young, 1999; Tennstedt *et al*., 2003).

A search for homologous sequences in the GenBank/EMBL databases revealed that similar gene cassettes are found worldwide in *Enterobacteriaceae* (*Escherichia*, *Salmonella*, *Klebsiella*, *Serratia*, *Yersinia)* and in other *Proteobacteria* (*Pseudomonas*, *Acinetobacter*) from animal and human isolates and from wastewater. The occurrence of integron-associated resistance gene cassettes on antibiotic resistance plasmids isolated from a wastewater treatment plant was studied by Tennstedt *et al*. (2003), who found eight different gene cassettes for aminoglycoside-modifying enzymes and seven gene cassettes for dihydrofolate reductases in class 1 integrons. These authors also found genes associated with resistance to other families of antibiotics, namely oxacillinase and chloramphenicol export protein. In a study on *Escherichia coli* isolated from animals with different levels of exposure to humans, Skurnik *et al*. (2006) found class 1 integrons, exhibiting predominantly the gene cassettes *aadA*1/*dfrA*1, mainly in animals living in close contact with humans. These authors concluded that integrons may represent an adaptive response to the exposure to antimicrobial pressure above a certain threshold. In the present study, it was possible to establish an association between the integron-specific regions and the respective resistance phenotypes. Nevertheless, these genetic elements did not account for the total resistance phenotype observed for *Escherichia* spp.

The results presented herein suggest an association between the resistance phenotypes to the antibiotics tetracycline, amoxicillin, sulfamethoxazole/trimethoprim and ciprofloxacin, which, on the basis of genetic analysis, cannot be attributed simply to the presence of a class 1 integron variable region. A similar association was presented by Leverstein-van Hall *et al*. (2003) who, in a study on clinical isolates of *Enterobacteriaceae*, reported that the combined resistance to both ampicillin and sulfamethoxazole/trimethoprim is the starting point for additional resistance acquisition, namely for other β-lactams, aminoglycosides, cephalosporins and ciprofloxacin. According to this model of multidrug resistance acquisition, the presence of integrons clearly associated with the sulfamethoxazole/trimethoprim resistance phenotype may represent an advantage. However, resistance to β-lactams was not observed within the variable regions of the class 1 integrons detected in the present study. The results presented suggest that, despite the dramatic difference in selective pressure effect imposed, a similar pattern of resistance acquisition may take place in hospital or environmental conditions. The elucidation of the nature of selective pressure factors and of the ecology and dynamics of these bacterial populations will contribute to a comprehensive understanding of the factors underlying the acquisition of antibiotic resistance.

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