Plasmid-mediated multiple antibiotic resistance of *Escherichia coli* in crude and treated wastewater used in agriculture

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ABSTRACT

A total of 273 *Escherichia coli* isolates from raw and treated municipal wastewaters were investigated to evaluate the frequency and persistence of antibiotic resistance and to detect the occurrence of conjugative R plasmids and integrons. The highest resistance rates were against ampicillin (22.71%), tetracycline (19.41%), sulfamethoxazole (16.84%) and streptomycin (14.28%). Multiple antibiotic resistance was present in 24.17% of the isolates. Several multiple antibiotic-resistant isolates proved to be able to transfer en bloc their resistance patterns by conjugative R plasmids with different molecular sizes and restriction profiles. Class 1 integrons of 1 or 1.5 kbp were found in 5 out of 24 representative multiresistant *E. coli* isolates. Although wastewater treatments proved to be effective in eliminating *Salmonella* spp. and in reaching WHO microbiological standards for safe use of wastewater in agriculture, they were ineffective in reducing significantly the frequency of plasmid-mediated multiple antibiotic resistance in surviving *E. coli*. Since multiple antibiotic-resistant bacteria carrying integrons and conjugative R plasmids can constitute a reservoir of antibiotic-resistance genes in wastewater reclaimed for irrigation, risks for public health should be considered. Bacterial strains carrying R plasmids and integrons could contaminate crops irrigated with reclaimed wastewater and transfer their resistances to the consumers' intestinal bacteria.

Key words | antibiotic resistance, *Escherichia coli*, integrons, R plasmids, wastewater

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INTRODUCTION

The worldwide increase in water consumption leads to the generation of more wastewater in urban areas and makes more important the use of reclaimed water for different purposes. Reclaimed wastewater can be used for different non-drinkable applications in industry and, especially, in agriculture. Wastewater used for irrigation must be submitted to treatments for the removal of pathogens in order to avoid health risks, mainly of enteric infections, for crop consumers, producers and handlers. According to the microbiological guidelines for safe use of wastewater in agriculture developed by the World Health Organization (WHO) and more recently revisited by Carr *et al.* (2004), less than 0.1 intestinal nematode eggs must be detected in 1 litre, while up to 1,000 faecal coliform bacteria per 100 ml can be tolerated for unrestricted irrigation. In the United States, much stricter wastewater quality standards for irrigation are recommended by the Environmental Protection Agency (Anon 1973) but, lacking federal standards for the quality of reclaimed water, individual states have developed guidelines mainly based on the daily monitoring of faecal coliform bacteria on a single, 100-ml sample, assuming a predictive relationship between indicator microorganisms and pathogen presence (Carr *et al.* 2004); however, new strategies in monitoring reclaimed water are required, because of the imperfect relationship between coliform bacteria and pathogens (Leclerc *et al.* 2001; Harwood *et al.* 2005). Further risks for public health could

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derive from the persistence in treated wastewater of *Escherichia coli* carrying conjugative R plasmids and integrons. Antibiotic-resistant *E. coli* surviving treatment processes enter the environment and can be ingested by people through contaminated crops. Experimental studies have shown that enteric pathogens can become antibiotic resistant by acquiring conjugative R plasmids from *E. coli* in the intestine (Poppe *et al.* 2005). However, there are relatively few available data on the prevalence of conjugative R plasmids and integrons in *E. coli* in wastewater used for irrigation (Ferreira da Silva *et al.* 2007).

The aims of the present study were: (i) to determine the frequency of antibiotic resistant *E. coli* in municipal wastewaters after treatments able to eliminate other pathogenic microorganisms (*Salmonella* spp. and nematode eggs); (ii) to verify the persistence of antibiotic resistances in the *E. coli* surviving in secondary treated effluents and in tertiary treated (polished) waters used in agriculture; (iii) to verify the transferability of resistance determinants of isolates exhibiting representative antibiotic resistance patterns; and (iv) to detect conjugative R plasmids and class 1 integrons in antibiotic-resistant isolates.

METHODS

Treatment plants

Two municipal wastewater treatment plants with an activated sludge system and different cleaning stages, located in two different towns (Caltagirone and San Michele di Ganzaria) in Sicily (Italy), were selected for the present investigation. Technical data for the two plants are shown in Table 1. The secondary effluent from biological treatment (activated sludge) in plant A was polished (tertiary treatment) by storage in a deep reservoir, while the secondary effluent from biological treatment (activated sludge) in plant B was polished (tertiary treatment) in a constructed wetland. Polished (tertiary) effluents from both plants are used for irrigation in agriculture.

Sample collection and processing

For this investigation, 108 sewage samples (11 litres each), 72 from plant A and 36 from plant B, were aseptically collected over a 1-year period, from January to December 2004, using sterile screw-capped bottles (1 litre) and tanks (10 litres). At treatment plant A, samples were collected every two weeks from the inflow (crude wastewater), from the secondary clarifier effluent (after biological treatment) and from the deep reservoir effluent (polished water), while at treatment plant B samples were collected every month from the inflow (crude wastewater), from the secondary clarifier effluent (after biological treatment) and from the constructed wetland (polished water). All samples were transported in a refrigerated state (+4°C) to the laboratory for immediate processing. The samples were analysed by standard membrane filtration technique (*Standard Methods* 1995) using 47-mm acetate membrane filters (Sartorius AG, Goettingen, Germany) with a nominal pore size of 0.45 μm. Samples from inflow and effluent from secondary treatment were filtered after dilution with 0.9% NaCl solution, while polished water samples were filtered both directly and after dilution. Filters were placed on the surface of plates of CHROMagar *E. coli* (CHROMagar, Paris, France), a chromogenous selective medium. After 24 h incubation at 37°C, plates were inspected for growth and two or three colonies per plate showing the typical characteristics of *E. coli* were selected for biochemical identification by the API 20E system (bioMérieux, Basingstoke, UK) and for antibiotic susceptibility testing.

Salmonellae were detected and quantified by a MPN (most probable number) procedure using Salmosyst broth (Merck, Darmstadt, Germany) as a liquid medium for both pre-enrichment and selective enrichment and Rambach agar (Merck) for plating according to a procedure for rapid detection and isolation of these bacteria (Pignato *et al.* 1995). In brief, one 50 ml and three 10 ml aliquots of double concentrated liquid medium were inoculated with an equal volume of water sample, while separate 10 ml aliquots of normal concentrated liquid medium were inoculated with 1 ml and 0.1 ml, respectively, and in triplicate. After 6 h of pre-enrichment at 37°C and 18 h of selective enrichment at 37°C, broth cultures were plated on Rambach agar and plates were incubated at 37°C. Red colonies that developed after 24 h of incubation were cultured on Kligler Iron Agar (Oxoid, Basingstoke, UK) and eventually submitted to full biochemical identification by the API 20E system and to serological identification by polyvalent and monovalent *Salmonella* anti-O and anti-H sera (either Diagnostics

Table 1 | Technical data for the treatment plants studied

	Plant A	Plant B
System	Activated sludge	Trickling filter
Population equivalent total capacity	40,000	6,000
Population equivalent actual capacity	35,000	5,000
Wastewater flow rate ($l\,s^{-1}$)	80	8
Wastewater	Municipal	Municipal
Mechanical stage	Coarse screen, sand and oil tank, primary sedimentation	Coarse screen, Imhoff tank
Biological stage	Aeration tank, secondary sedimentation	Secondary sedimentation
Aeration system	Surface aerators (low speed)	–
Polishing stage	Storage reservoir (flow rate only about $30\,l\,s^{-1}$)	Constructed wetland (flow rate only about $2\,l\,s^{-1}$)
Reclaimed wastewater use	Agriculture	Agriculture

Pasteur, Marnes-La-Coquette, France or Biogenetics Diagnostics, Padua, Italy).

Nematode eggs were detected in 10 l volumes according to the procedure recommended by Ayres & Mara (1996).

Antibiotic susceptibility testing

E. coli isolates were screened for resistance to ten antibiotics (Sigma-Aldrich, Gillingham, UK) by streaking a loopful of 18 h broth cultures on Mueller-Hinton Agar (Bio-Rad SA, Marnes-la-Coquette, France) plates with ampicillin (A) $32\,\mu g\,ml^{-1}$, cephalothin (Ce) $16\,\mu g\,ml^{-1}$, streptomycin (S) $64\,\mu g\,ml^{-1}$, kanamycin (K) $64\,\mu g\,ml^{-1}$, gentamicin (G) $16\,\mu g\,ml^{-1}$, chloramphenicol (C) $32\,\mu g\,ml^{-1}$, tetracycline (T) $16\,\mu g\,ml^{-1}$, sulfamethoxazole (Su) $512\,\mu g\,ml^{-1}$, trimethoprim (Tmp) $10\,\mu g\,ml^{-1}$ and nalidixic acid (NA) $64\,\mu g\,ml^{-1}$. Plates were incubated for 24 h at 37°C and isolates that yielded bacterial growth were recorded as resistant to the corresponding antibiotic. A selected number of ampicillin resistant isolates exhibiting representative antibiotic resistance patterns were tested by the disk diffusion method on Mueller-Hinton Agar according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (Soussy *et al.* 2000).

Resistance transfer determination

Resistance transfer was tested for 24 ampicillin-resistant *E. coli* isolates from inflow, secondary effluent and polished waters exhibiting representative antibiotic resistance patterns. *E. coli* C1a (NalA) resistant to nalidixic acid was used as the recipient strain. Equal volumes (1 ml and 1 ml) of broth cultures of donor and recipient strains at logarithmic phase were mixed and incubated for 18 h at 37°C. Transconjugants were selected on Mueller-Hinton Agar supplemented with ampicillin ($100\,\mu g\,ml^{-1}$) and nalidixic acid ($64\,\mu g\,ml^{-1}$). Transfer frequencies were calculated as the numbers of resistant recipient bacteria arising per resistant donor, given as the means of duplicate experiments.

Plasmid analysis

Plasmid DNA was purified from transconjugants by an alkaline lysis procedure (Takahashi & Nagano 1984) and subjected to 0.8% agarose gel electrophoresis in TBE 0.5x at 100 V for 1 h. The molecular size of plasmids was determined with Taxotron software (Institut Pasteur, Paris, France) by reference to plasmids of known size (RP4, 54 kp and pIP173, 126 kp) mixed with a supercoiled DNA ladder (Invitrogen, Carlsbad, California). Plasmid DNA was cleaved with *Eco* RI (Roche, Mannheim, Germany) and electrophoresed in 1% agarose gel at 30 V for 16 h for restriction fragments length polymorphism analysis.

PCR amplification

Total DNA was extracted using the InstaGene Matrix kit (Bio-Rad) in accordance with the manufacturer's

recommendations. Amplification of the gene cassette of the class 1 integrons was performed by using 5′-CS (5′-GGCATCCAAGCAGCAAGC-3′) and 3′-CS (5′-AAGCAGACTTGACCTGAT-3′) primers (Lévesque *et al*. 1995). Amplifications were performed on 50 μl volumes containing DNA (2.5 μl), primers (50 pmol each), deoxynucleoside triphosphate (200 LM), *Taq* DNA polymerase (1.25 U Ampli *Taq* Gold; Roche) and its buffer, $MgCl_2$ (2 mM), and dimethyl sulfoxide (10%). The cycling conditions included 10 min of denaturation at 94°C (1 cycle) and 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 2 min of polymerization at 72°C (30 cycles), followed by 10 min of extension at 72°C.

Data analysis

Data comparison was performed using the Chi-square test ($p < 0.05$) in the statistical package for Social Science–Statistical Program, SPSS software version 14.0 (SPSS© Inc., Chicago, Illinois).

RESULTS AND DISCUSSION

Concentrations of *E. coli*, *Salmonella* spp. and nematode eggs in inflow crude sewage, effluent from secondary clarifiers and in polished water from the two sewage reclamation facilities are shown in Table 2. Although *E. coli* showed a 2 log reduction in the secondary effluent of both plants and a further 2 log reduction in the polished waters, surviving *E. coli* reached the receiving soils. *Salmonella* spp. belonging to different serovars and nematode eggs were commonly detected in crude wastewater inflows and occasionally in the secondary effluents but they were never detected in polished waters of both treatment plants.

A total of 273 *E. coli* isolates were investigated with regard to their antibiotic resistances: 177 from plant A (59 from inflow, 59 from secondary effluent, and 59 from polished water) and 96 from plant B (32 from inflow, 32 from secondary effluent, and 32 from polished water). Out of the 273 isolates, 194 (71.06%) were sensitive to all tested antibiotics, while 13 (4.76%) were resistant to one, 25 (9.15%) to two, 8 to three (2.93%), and 33 (12.08%) to four or more antibiotics. The majority of multiresistant isolates were resistant to ampicillin: 19 out of 25 resistant to two, seven out of eight resistant to three, and 33 out of 33 resistant to four or more antibiotics. The highest resistance rates were against ampicillin (22.71%), tetracycline (19.41%), sulfamethoxazole (16.84%) and streptomycin (14.28%), followed by trimethoprim (9.15%), nalidixic acid (8.00%), chloramphenicol (5.12%) and kanamycin (2.93%). Intermediate resistance to cephalotin (16–32 $\mu g\,ml^{-1}$) was exhibited by 15.75% of isolates. No isolates resistant to gentamicin were found. In both plants, frequency of isolates resistant to one or more antibiotics decreased from 36.26% in crude wastewater inflows to 24.17% in polished waters for irrigation. The decrease was more evident in plant B (from 43.8% to 21.9%) than in plant A (from 32.2% to 25.4%). The percentage of isolates from the two treatment plants that were resistant to the antibiotics tested is shown in Table 3. A general decrease of resistant isolates was observed, mainly comparing crude with secondary effluent isolates, but the differences were not statistically significant.

As resistance to ampicillin was detected in the majority of the strains, ampicillin-resistant isolates from plant A (17 isolates) and plant B (7 isolates), representative of different antibiotic resistance patterns, were investigated for the transferability of their resistance determinants. Thirteen isolates (54.0%) proved to be able to transfer their antibiotic resistances at low frequencies (mean $= 1.2 \times 10^{-5}$). Eight isolates from plant A and three isolates from plant B transferred their resistance patterns en bloc to *E. coli* C1a, while one isolate from plant A transferred its resistances to ampicillin, sulfamethoxazole, trimethoprim, streptomycin but did not transfer chloramphenicol resistance, and one isolate from plant B which was resistant to ampicillin, sulfamethoxazole, trimethoprim, streptomycin, and tetracycline transferred only resistances to ampicillin and streptomycin (Table 4). According to the transferred resistances, transconjugants exhibited high levels of resistance like the donor strains. The minimum inhibitory concentrations (MICs) of ampicillin, streptomycin, kanamycin, tetracycline, and sulfonamides were 256 $mg\,l^{-1}$, while the MIC of trimethoprim was 32 $mg\,l^{-1}$. No resistances to third generation cephalosporins were exhibited by the ampicillin resistant donor and recipient strains.

Plasmid extraction from ten transconjugants revealed the presence of single plasmids with a size of 125 kb

Table 2 | *Escherichia coli* (CFU 100 ml^{-1}), *Salmonella* (MPN 100 ml^{-1}) and nematode eggs (per litre) in crude wastewater inflows, effluent from secondary clarifiers (biological treatment) and polished waters from two municipal wastewater treatment plants

	Plant A			Plant B		
	Mean	Min	Max	Mean	Min	Max
Crude influent						
E. coli	8.0×10^6	2.4×10^6	1.5×10^7	1.5×10^7	2.9×10^6	3.0×10^7
Salmonella	3.9×10^2	2×10^0	1.1×10^3	1.8×10^2	<1	1.1×10^3
Nematode eggs	2.7×10^1	2×10^0	5.0×10^1	1.7×10^2	<0.1	5.0×10^1
Secondary effluent						
E. coli	6.5×10^4	1.4×10^4	1.8×10^5	1.0×10^5	1.5×10^4	1.8×10^5
Salmonella	8×10^0	<1	3.9×10^1	2×10^0	<1	1.6×10^1
Nematode eggs	4×10^0	<0.1	2.0×10^1	7×10^0	<1	1.8×10^1
Polished water						
E. coli	8.5×10^2	2.5×10^1	3.3×10^3	9.9×10^2	2.9×10^1	5.0×10^3
Salmonella	<1	<1	<1	<1	<1	<1
Nematode eggs	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

(transconjugants from isolates BR21, BR23, BR41, BR80, BR81, DE3, DE45 and RA59), 54 kb (transconjugant from isolate BR51) and 60 kb (transconjugant from isolate RA70). Restriction fragments length polymorphism analysis by agarose gel electrophoresis of plasmid DNA cleaved with *Eco* RI produced nine different fingerprints (Figure 1). The conjugative plasmids from BR80 and BR81 had indistinguishable fingerprints, while the conjugative plasmids from BR23 and DE3 had very similar fingerprints (Figure 1). Strains BR80 and BR81 were isolated from the same sample of crude wastewater inflow from plant B, while strains DE3 and BR23 were isolated from samples collected from plant A in treated effluent in January 2004 and in crude inflow in April 2004, respectively.

Five of the 24 ampicillin-resistant isolates harboured class 1 integrons of 1.0 kb (two isolates from plant A) and 1.5 kb (two isolates from plant A and one isolate from plant B). Although both the municipal wastewater treatment plants studied were demonstrated to be able to reduce the number of pathogenic microorganisms in polished waters to levels allowing unrestricted crop irrigation according to the WHO guidelines (Carr *et al.* 2004) (i.e. less than 0.1 nematode eggs per litre and *E. coli* counts not exceeding 10^3/100 ml on a yearly mean), a high frequency of multiple

Table 3 | Prevalence of antibiotic resistance markers in *E. coli* isolates from the two treatment plants

		Resistance prevalence (%)												
Wastewater	No. of isolates	A	Su	Tmp	S	T	C	K	G	Ce	NA	R2	R3	R4
Plant A	177													
Crude	59	25.4	16.9	8.5	13.6	22.0	5.1	3.4	0.00	22.0*	5.1	25.4	18.6	13.5
Secondary effluent	59	20.3	20.3	11.9	16.9	16.9	10.2	5.1	0.00	20.3*	8.5	22.0	15.2	11.9
Polished water	59	18.6	13.6	8.5	15.3	16.9	5.1	1.7	0.00	20.3*	6.8	15.2	10.2	10.2
Plant B	96													
Crude	32	43.7	21.9	12.5	25.0	40.6	3.1	0.00	0.00	21.9*	25.0	34.4	28.1	25.0
Secondary effluent	32	15.6	12.5	6.2	3.1	12.5	0.00	6.2	0.00	28.1*	6.2	15.6	9.4	3.1
Polished water	32	15.6	15.6	6.2	9.4	9.4	3.1	0.00	0.00	21.9*	0.00	9.4	9.4	9.4

*Intermediate resistance (16–32 μg ml^{-1}).

A = ampicillin, Su = sulphamethoxazole, Tmp = trimethoprim, S = streptomycin, T = tetracycline, C = chloramphenicol, K = kanamycin, G = gentamicin, Ce = cephalothin, NA = nalidixic acid, R2 = resistant to two or more antibiotics, R3 = resistant to three or more antibiotics, R4 = resistant to four or more antibiotics.

Table 4 | Antimicrobial resistance patterns, resistance markers transferred to *E. coli* C1a, and class 1 integrons of representative ampicillin-resistant *E. coli* isolates

Isolate (date of isolation)	Resistance pattern	Resistance markers transferred to *E. coli* C1a	Integron PCR size (kb)
Plant A			
DE 43 (25.5.04)	A	No transfer	ND
BR 41 (25.5.04)	A T	A T	ND
BR 35 (18.5.04)	A T K	A T K	ND
RA 3 (27.1.04)	A S T	No transfer	ND
RA 10 (24.2.04)	A Su T	No transfer	ND
BR 76 (9.11.04)	A Su T	No transfer	ND
BR 77 (9.11.04)	A Su T	No transfer	ND
BR 52 (29.6.04)	A Su S	No transfer	ND
DE 18 (30.3.04)	A Su C	A Su C	1
BR 75 (9.11.04)	A Su S C	No transfer	1
BR 21 (6.4.04)	A Su Tmp T	A Su Tmp T	1.5
DE 3 (27.1.04)	A Su Tmp S T	A Su Tmp S T	1.5
BR 23 (13.4.04)	A Su Tmp S T	A Su Tmp S T	ND
BR 51 (29.6.04)	A Su Tmp S C	A Su Tmp S	ND
DE 45 (15.6.04)	A Su Tmp S T C	A Su Tmp S T C	ND
RA 82 (23.11.04)	A Su Tmp S T K	A Su Tmp S T K	ND
BR 5 (27.1.04)	A Su S T C K	No transfer	ND
Plant B			
BR 80 (15.11.04)	A T	A T	ND
BR 81 (15.11.04)	A T	A T	ND
DE 86 (30.11.04)	A T	No transfer	ND
BR 79 (15.11.04)	A Su S C	No transfer	ND
RA 56 (30.3.04)	A Su S T C	No transfer	ND
RA 59 (26.7.04)	A Su Tmp S	A Su Tmp S	1.5
RA 70 (13.9.04)	A Su Tmp S T	A S	ND

A = ampicillin, Su = sulphamethoxazole, Tmp = trimethoprim, S = streptomycin, T = tetracycline, C = chloramphenicol, K = kanamycin, ND = none detected.

antibiotic resistant strains was found among *E. coli* isolates surviving the treatment processes.

Previous studies on antibiotic resistance of *E. coli* isolates from wastewater have produced rather variable results, although high rates of resistances have been reported for several antibiotics, particularly against ampicillin, tetracycline and chloramphenicol (Krcmery *et al.* 1989; Tamanai-Shacoori *et al.* 1995; Goni-Urriza *et al.* 2000; Reinthaler *et al.* 2003). In our study, the highest resistance rates were to ampicillin, tetracycline and sulphonamides, while a relatively low frequency of chloramphenicol resistance was found, possibly in relation to a limited use of this antibiotic in Italy in recent years.

The fate of resistant bacteria in the course of the wastewater treatment processes and in the environment is questioned. In some studies, it was observed that the percentages of resistant *E. coli* and coliform isolates were decreasing during the treatment processes and, in most cases, were lower in the recipient river water than in the wastewater effluent (Gonzalo *et al.* 1989; Iwane *et al.* 2001; Guardabassi et al. 2002), leading to the conclusion that strains carrying antibiotic resistance factors have lower survival rates than susceptible bacteria in the environment. In contrast, Reinthaler *et al.* (2003), studying *E. coli* isolates from sewage, sludge and receiving waters, did not find a significant decrease in the resistance rates in the course of the treatment processes,

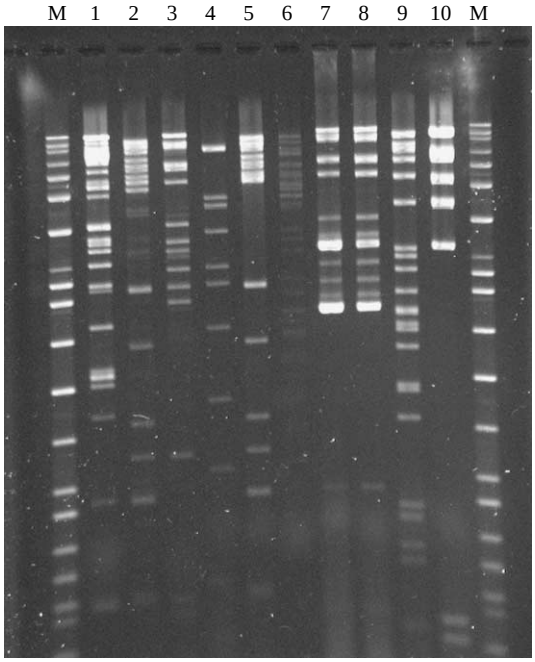

Figure 1 | Agarose gel electrophoresis of plasmid DNA cleaved with *Eco* RI. Lane M, Raoul wide-spectrum marker (Qbiogene, Heidelberg, Germany); lane 1, strain BR21; lane 2, strain BR23; lane 3, strain BR41; lane 4, strain BR51, lane 5, strain DE3; lane 6, strain DE45; lane 7, BR80; lane 8, strain BR81; lane 9, strain RA59; lane 10, strain RA70.

while Ferreira da Silva *et al.* (2007) observed a generalized increase in antibiotic resistance in *Escherichia* spp. isolates from the crude wastewater influent to the treated effluent, although a similar frequency of class 1 integrons was found. Our results showed a reduction of the percentages of resistant and multiple resistant *E. coli* isolates in the course of the processes from crude wastewaters to polished waters, mainly from crude wastewaters to secondary effluents. Although the differences were not significant at the $p < 0.05$ level, the lack of significance may reflect the small size of the sample, rather than the reality of the trend. However, even if the treatment processes were efficient in reducing the number of pathogens enough to fulfil the WHO guidelines for the safe use of wastewaters in agriculture, a high proportion of *E. coli* isolates with multiple antibiotic resistance was found to survive in polished waters from both plants.

Multiple antibiotic-resistant bacteria can spread their antibiotic-resistance genes to susceptible strains of the same species or to other species or genera by different mechanisms, mainly by conjugative R plasmids (Opal & Medeiros 2005). Mispagel & Gray (2005) showed that wastewater oxidation ponds discharged high numbers of antibiotic resistant bacteria containing transferable plasmids, surviving the treatment process. We also studied the capacity of *E. coli* isolates from crude wastewaters, secondary effluents and polished waters to transfer their antibiotic resistances. The use of a highly mutated laboratory recipient strain (*E. coli* C1a) probably determined transfer frequencies lower than those observed using natural isolates (Mach & Grimes 1982). Although the frequencies of transfer were low in our experiments, in natural conditions bacteria could effect significant transfer of their genetic materials in the absence of antibiotics as selective agents (Mach & Grimes 1982; Kruse & Sørum 1994; Marcinek *et al.* 1998).

Genetic analysis demonstrated that the conjugative antibiotic-resistant isolates carried a variety of R plasmids which could be differentiated on the basis of their molecular size and restriction profiles. Furthermore, several isolates harboured class 1 integrons, known to be able to capture genes, notably those encoding antibiotic resistance. The interest in epidemiological studies of the role of integrons in the spread of antibiotic resistance among bacterial populations has recently been stressed (Norrby 2005). The frequency of integrons is high in Gram-negative clinical isolates, up to 57% for class 1 integrons in *E. coli* isolates (Rao *et al.* 2006). The percentage of our multiresistant *E. coli* harbouring integrons was lower than that reported for clinical isolates, but exceeded that recently published by Ferreira da Silva *et al.* (2007), who detected integron-specific regions, with sizes in the range 1–1.5 kbp or more, in 10% and 9.6% of *E. coli* spp. isolates from crude and treated wastewater, respectively. However, the selection of our isolates on the basis of their ampicillin resistance may have led to an overestimation of these genetic elements.

CONCLUSIONS

Although wastewater treatments proved to be effective in reaching WHO microbiological standards for safe use of wastewater in agriculture, they were ineffective in reducing significantly the frequency of plasmid-mediated multiple antibiotic resistance in surviving *E. coli*. Furthermore, the presence of a variety of conjugative plasmids and integrons was detected in the multiresistant *E. coli* isolates. These data contribute to raise questions on the importance of multi-

resistant *E. coli* carrying conjugative R plasmids and integrons as a reservoir of antibiotic-resistance genes in wastewater, as well as on the possible role of contaminated crops in disseminating antibiotic-resistant enteric bacteria when they are irrigated with reclaimed wastewater.

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