Resistance patterns of *Escherichia coli* isolated from sewage sludge in comparison with those isolated from human patients in 2000 and 2009

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ABSTRACT

For some time now, antibiotic-resistant bacterial strains have been found in the human population, in foods, in livestock and wild animals, as well as in surface waters. The entry of antibiotics and resistant bacterial strains into the environment plays an important role in the spread of antibiotic resistance. The goal of the present study was to monitor the entry of antibiotic resistances into the environment through the contamination of wastewater. To assess the extent of transmission of antibiotic resistances from human sources into the environment, the resistance patterns of *Escherichia coli* strains isolated from human patients have been compared to those found in strains isolated from sewage sludge. Our results may indicate if resistances to particular antibiotics are more prone than others to spread into the environment. To monitor the increase of specific resistances over time, samples taken in the years 2000 and 2009 were analysed. Our study shows that for some antibiotics a parallel development of resistance patterns has taken place in both patient and environmental samples over time. For other sets of antibiotics, independent developments have occurred in the samples. A clear increase of multi-resistant *E. coli* strains over time was observed in samples from both sources.

Key words | antibiotic resistance, Austria, *Escherichia coli*, infection, wastewater

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INTRODUCTION

The rise and spread of antibiotic-resistant bacteria has been a common problem since the day treatment with antibiotics started (Aminov & Mackie 2007; Woodford & Livermore 2009). Resistant pathogens have increasingly spread beyond hospitals and other medical institutions. There are reports of (multi-)resistant bacteria such as MRSA (methicillin-resistant *Staphylococcus aureus*) and ESBL (extended spectrum beta-lactamase-producing *Enterobacteriaceae*) in healthy humans with no direct connection to medical institutions, in food and even in wild animals (Carattoli 2008; Ghidan *et al.* 2008; Pitout & Laupland 2008; Grisold *et al.* 2009; Poeta *et al.* 2009).

This development is further facilitated by the widespread use of (broad-spectrum) antibiotics in human and veterinary medicine and agriculture. In populated regions, contact with antibiotics seems to be nearly inescapable for bacteria. Antibiotics can be found in nearly all surface waters (Kummerer 2009a, b). Some antibiotics can even be found in ground water as deep as 10 m and more (Batt *et al.* 2006).

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The presence of antibiotics in the environment leads to the selection of antibiotic-resistant bacteria outside as well as within the hospital setting (Yang & Carlson 2003; Kummerer 2009a, b; Zhang *et al.* 2009). Moreover, there is also direct input of antibiotic-resistant bacteria from areas with a high concentration of such strains, e.g. due to low efficiency treatment of wastewater. Studies from both European and non-European countries report that hospital and urban wastewater and sewage sludge can show high levels of antibiotic-resistant bacteria. Furthermore, both in raw sewage and in the effluent of treatment plants, the proportion of antibiotic-resistant bacteria has increased in recent years (Goni-Urriza *et al.* 1999; Reinthaler *et al.* 2003; Silva *et al.* 2006; Prado *et al.* 2008; Yang *et al.* 2009).

As we reported in a recent study, *Escherichia coli* can even survive some of the sewage sludge treatment procedures applied in Austria (Reinthaler *et al.* 2010). This opens the possibility for resistant strains to re-enter the food chain via treated wastewater or sewage sludge which is applied to arable land. Hence, the analysis of both patient and environmental samples is important to understand the routes of transmission of antibiotic resistance from humans to the environment and, ultimately, back to humans (Teuber 1999; Salyers 2002; Reinthaler *et al.* 2010; Dhanji *et al.* 2011).

The aim of the present study was to investigate the changes in the resistance patterns of *E. coli* isolates from infected outpatients and from activated sewage sludge for clinically relevant antibiotics between 2000 and 2009. The investigation was designed not only to compare *E. coli* isolates from different sources, but also to observe changes in resistance over time and to look for indications of interdependency of resistance patterns observed in isolates from human and environmental sources.

MATERIALS AND METHODS

Sampling

Sewage sludge isolates

The first set of sewage sludge (activated sludge) samples was collected between April and September 2000. Sewage sludge samples were collected from different sewage treatment plants on five sampling days each and 155 different *E. coli* strains were isolated. The second set of samples was collected between April and September 2009 on six sampling days. In this second sampling period, 201 different *E. coli* strains were isolated. All sewage treatment plants are located in Styria (Austria) in the area around the city of Graz. Only sewage treatment plants which did not receive wastewater from hospitals were used for this study.

The samples were taken from activated sewage sludge. The aerobic degradation of organic matter takes place in the activated sludge tank. The activated sludge is an accumulation of bacteria, organic detritus and ciliates, which form activated sludge flakes in wastewater.

The sludge samples were collected using sterile wide-mouth bottles. They were transported to the laboratory in a cooler and immediately stored in a refrigerator at 4–8 °C for up to 24 hours until processing.

Human isolates

Human isolates were randomly picked out of a collection of *E. coli* isolates from different sources (urine, sputum and stool samples, wound, skin and respiratory tract swabs) which had been sent to the diagnostic laboratory of the Medical University Graz (Austria) by general medical practitioners. In both years, 2000 and 2009, 500 single first isolates of *E. coli* were collected between April and September.

Laboratory tests

Sewage sludge samples were screened for *E. coli* on Chromocult Agar (Merck, Darmstadt, Germany) according to Reinthaler *et al.* (2010). Human strains were isolated on Endo Agar (OXOID, Cambridge, UK). *E. coli* strains were identified using Vitek 2 (Testcard: ID-GNB, bioMerieux, Vitek, Inc., Hazelwood, MO, USA). The isolated *E. coli* strains were tested for their resistance patterns against the antibiotics listed in Table 1. For 15 antibiotics (amoxicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefalotin, cefuroxime-axetil, cefoxitin, cefotaxime, ceftazidime, meropenem, gentamicin, amikacin, trimethoprim-sulfamethoxazole, nitrofurantoin, norfloxacin, ciprofloxacin),

Table 1 | Comparison of resistant isolates 2000 versus 2009: percentage of the resistant isolates (resistant plus intermediate resistant isolates) to antibiotics tested in 2000 and 2009. The proportion of isolates which were tested as intermediate resistant is given in parentheses

	E. coli from human			*E. coli* from sewage sludge		
	2000[a]	2009[a]	*P*-value	2000[b]	2009[c]	*P*-value
Penicillins						
Amoxicillin	22.8% (0.4%)	35.2% (1.6%)	<0.01	4.5% (0.6%)	12.4%	0.02
Amoxicillin/clavulanic acid	3.6% (2.4%)	10.8% (7.8%)	<0.01	2.5% (1.9%)	2% (0.5%)	0.99
Piperacillin/tazobactam	0%	1.2% (0.6%)	0.04	0%	0%	1
Cephalosporins						
Cefalotin	10.4% (6.4%)	24.4% (18.6%)	<0.01	15.5% (12.9%)	21.9% (17.9%)	0.16
Cefuroxime-axetil	0%	7.6% (2.8%)	<0.01	1.9% (1.9%)	5% (4.5%)	0.22
Cefoxitin	0%	2.2% (1.4%)	<0.01	1.9% (1.9%)	0%	0.16
Cefotaxime	0%	4.6%	<0.01	0%	0.5%	0.2
Ceftazidime	0%	4.6%	<0.01	0%	0.5%	0.2
Carbapenems						
Meropenem	0%	0%	1	0%	0%	1
Aminoglycosides						
Gentamicin	0.6%	4.2% (0.2%)	<0.01	0.6%	3%	0.23
Amikacin	0%	0.6% (0.2%)	0.25	0%	0%	1
Quinolones						
Ciprofloxacin	4.2%	14.2% (0.4%)	<0.01	0%	2.5%	0.13
Norfloxacin	5.4%	16.6% (0.4%)	<0.01	0%	2.5%	0.13
Others						
Nitrofurantoin	1.4% (0.4%)	1.8% (1.2%)	1	0.6% (0.6%)	2.5% (2.5%)	0.36
Trimethoprim-sulfamethoxazole	12.8%	21.6%	<0.01	0.6%	8.5%	<0.01

[a] $n = 500$.
[b] $n = 155$.
[c] $n = 201$.

resistance was also tested using Vitek 2 (Testcard: AST-N020) according to guidelines of the Clinical and Laboratory Standards Institute (CLSI) criteria. The McFarland standard was 0.55–0.62. For quality control of Vitek analyses, *E. coli* ATCC 25922 was used. Strains were identified as susceptible, intermediate resistant or resistant according to CLSI criteria. Intermediate resistance and resistance are subsumed as resistance in the analyses.

Statistics

The statistical evaluations were carried out using R® Version 2.12, a free software environment for statistical computing and graphics (www.r-project.org). Numbers of susceptible and resistant *E. coli* were compared. Group specific proportions were tested on their equality by a two-sided binomial test; *p*-value smaller than 0.05 was considered to indicate statistical significance.

RESULTS

E. coli strains isolated from patients and sewage sludge in 2000 and 2009 were investigated for their antibiotic resistance patterns. The quantitative evaluation of these data is presented in Table 1, which compares antibiotic resistances in 2000 and 2009, and Table 2, which focuses on differences between the two sample types.

All *E. coli* strains isolated from patients treated in 2000 were sensitive to seven of the 15 tested antibiotics,

Table 2 | Comparison of resistant isolates from human versus sewage sludge: percentage of the resistant isolates to 15 antibiotics tested, comparison between *E. coli* isolates of human origin and from sewage sludge

	E. coli from year 2000			*E. coli* from year 2009		
	Human[a]	Sewage sludge[b]	*P*-value	Human[a]	Sewage sludge[c]	*P*-value
Penicillins						
Amoxicillin	22.8%	4.5%	<0.01	35.2%	12.4%	<0.01
Amoxicillin/clavulanic acid	3.6%	2.5%	0.72	10.8%	2%	<0.01
Piperacillin/tazobactam	0%	0%	1	1.2%	0%	0.26
Cephalosporins						
Cefalotin	10.4%	15.5%	0.13	24.4%	21.9%	0.54
Cefuroxime-axetil	0%	1.9%	0.02	7.6%	5%	0.28
Cefoxitin	0%	1.9%	0.02	2.2%	0%	0.07
Cefotaxime	0%	0%	1	4.6%	0.5%	0.02
Ceftazidime	0%	0%	1	4.6%	0.5%	0.02
Carbapenems						
Meropenem	0%	0%	1	0%	0%	1
Aminoglycosides						
Gentamicin	0.6%	0.6%	1	4.2%	3%	0.59
Amikacin	0%	0%	1	0.6%	0%	0.64
Quinolones						
Ciprofloxacin	4.2%	0%	0.02	14.2%	2.5%	<0.01
Norfloxacin	5.4%	0%	<0.01	16.6%	2.5%	<0.01
Others						
Nitrofurantoin	1.4%	0.6%	0.74	1.8%	2.5%	0.77
Trimethoprim-sulfamethoxazole	12.8%	0.6%	<0.01	21.6%	8.5%	<0.01

[a] $n = 500$.
[b] $n = 155$.
[c] $n = 201$.

i.e. piperacillin/tazobactam, cefuroxime-axetil, cefoxitin, cefotaxime, ceftazidime, meropenem and amikacin. Resistances occurred particularly to amoxicillin (22.8% resistant strains) and cefalotin (10.4%) (Table 1).

Notably, in 2009, the proportion of resistant strains to most of the tested antibiotics increased, with the only antibiotic remaining effective against all isolates being meropenem. For 12 of the tested antibiotics, a significant increase of resistant strains was observed, with $p < 0.05$. Only for amikacin and nitrofurantoin was there no statistically significant ($p > 0.05$) increase of resistant strains (Table 1).

When looking at antibiotic resistances in *E. coli* isolated from sewage sludge in 2000, the two most frequent resistances (amoxicillin and cefalotin) were the same as recorded for patient samples. Nevertheless, amoxicillin resistance rates were significantly lower in sewage sludge than in human samples (Table 1). Significant differences between human and sewage sludge samples were also seen for trimethoprim-sulfamethoxazole and the two quinolones, ciprofloxacin and norfloxacin, to which resistances were only found in human samples from 2000, but not in sewage sludge samples. Most of the antibiotics with no or low proportions of resistant strains occurring in human samples in 2000 (amoxicillin/clavulanic acid, piperacillin/tazobactam, cefotaxime, ceftazidime, meropenem, gentamicin, amikacin and nitrofurantoin) also showed no or low proportions of resistances in sewage sludge samples ($p > 0.05$). Exceptions to that were three strains from the sewage sludge, which showed intermediate resistance against cefuroxime-axetil and cefoxitin (Tables 1 and 2).

In 2009, amoxicillin and cefalotin resistances were still the most frequently found resistances in sewage sludge samples, although a significant increase of resistant strains over time was only observed for amoxicillin ($p = 0.02$). The second antibiotic with a significant increase of resistant strains in sludge samples between 2000 and 2009 was trimethoprim-sulfamethoxazole ($p < 0.01$). Increased occurrences of resistances were also observed for cefalotin, cefuroxime-axetil, cefotaxime, ceftazidime, gentamicin, nitrofurantoin, norfloxacin and ciprofloxacin, although these increases were not statistically significant.

When comparing resistances in 2009 from the two different sample sets, the overall picture is similar to that in 2000. Similarly low resistance rates in human and environmental samples were found for piperacillin/tazobactam, cefuroxime-axetil, cefoxitin, meropenem, gentamicin, amikacin and nitrofurantoin ($p > 0.05$ when comparing resistance rates in human and sewage sludge samples in 2009). Also similarly to 2000, amoxicillin resistances are frequently found in sewage sludge from 2009, but still to a significantly lower extent than in human samples ($p > 0.01$). In contrast, however, resistance to three antibiotics (amoxicillin/clavulanic acid, cefotaxime and ceftazidime) increased in human samples only (Table 2).

Generally, the sludge samples showed a significant decrease ($p < 0.01$) of those strains which were susceptible to all antibiotics, namely from 81.3% to 65.7%. The proportion of strains which were resistant to two or more antibiotics rose from 5.2% to 12.4%. No isolates from 2000 showed more than four resistances; in 2009, however, the maximum number of resistances was nine (to antibiotics from five different classes) (Table 3).

Table 3 | Number of antibiotics per individual *E. coli* strain from sewage sludge tested as resistant

Resistances to antibiotic	2000 ($n = 155$) Number of isolates	% of isolates	2009 ($n = 201$) Number of isolates	% of isolates
0	126	81.29%	132	65.67%
1	21	13.55%	44	21.89%
2	4	2.58%	14	6.97%
≥3	4	2.58%	11	5.47%

DISCUSSION

Communal wastewater is a pool for resistant bacteria, as well as antibiotics, of human origin. Hence, the presence of antibiotic resistances in wastewater should, at least in part, be a reflection of the situation in the human population. The investigation of antibiotic resistances in bacteria in wastewater is important to obtain an understanding as to which resistances are more prone than others to escape into the environment. This knowledge is of special importance as pathogens in the environment can be a source for new infections of humans. The intention of the present study was to follow developments in both the environment and the human population with respect to the occurrence of antibiotic resistances. We analysed activated sludge as an example of a wastewater source.

The current study shows that the trend found in the resistance patterns from the medical environment is largely reflected in the sludge samples. What causes concern is not only the general increase in resistance in the environment, which has been observed for the last two decades, but also especially the fact that multi-resistant bacteria are increasingly able to survive in the environment. These data are consistent with findings in several studies regarding the occurrence of multi-resistant strains in various surface waters (Kummerer 2004; Pignato *et al.* 2009; Zhang *et al.* 2009; Luczkiewicz *et al.* 2010; Reinthaler *et al.* 2010).

In general, most resistances found in human samples were, both in 2000 and 2009, at least to some extent also found in sewage sludge samples. An increased occurrence of resistances in 2009 in comparison to 2000 was similarly obvious in the human and sewage sludge samples.

Despite these parallels, some interesting differences in the developments of resistance levels from 2000 to 2009 can be observed between human and sewage sludge samples. In this context, it has to be considered that the investigated human strains originated from patients under medical treatment. As patient strains have likely been exposed to antibiotics more frequently than strains from healthy people, an increased occurrence of antibiotic resistances is expected in patient samples. This is a problem of most studies in this field, which usually investigate antibiotic resistances in patient samples and hence likely result in higher antibiotic resistance rates than expected in the total

population. Unfortunately, no direct comparisons between the healthy population and treated patients have been undertaken. Furthermore, there are no data on antibiotic resistances of *E. coli* in the healthy Austrian population. An example is the fluoroquinolone resistance in 2000, which was the only resistance that was not detected at all in the sewage sludge samples and showed significantly higher resistance levels (ca. 5%) in human samples. In addition, the resistance rates of human isolates to fluoroquinolone had increased between 2000 and 2009, but quinolone resistances were nevertheless found only rarely in sewage sludge samples in both years.

Notably, the percentage of strains resistant to fluoroquinolone in sewage sludge samples determined in this study are in the mid-range compared to data from sewage sludge and wastewater from recent studies (0–10.5% resistant strains) (Holzel *et al.* 2010; Lu *et al.* 2010; Luczkiewicz *et al.* 2010).

In contrast, however, to our study, Luczkiewicz *et al.* (2010) who compared their data from wastewater (10.5% resistances) to the EARSS (European Antimicrobial Resistance Surveillance System) data, found no significant difference between human and wastewater samples concerning quinolone resistance.

Similarly to quinolones, trimethoprim/sulfamethoxazole resistances occurring in human patients in 2000 were relatively weakly reflected in sewage sludge samples, with only 0.6% resistances in sewage sludge isolates as compared to 12.8% in human isolates (thereby being the second highest resistance rate of human samples in 2000). In contrast, however, to quinolones, trimethoprim/sulfamethoxazole resistance became one of the top resistances in the isolates from sewage sludge in 2009. This is especially remarkable because the prescription of trimethoprim/sulfamethoxazole decreased between 2000 and 2009 in Austria (Metz-Gercek *et al.* 2009).

In the same time period, however, the use of penicillins in combination with beta-lactamase inhibitors like clavulanic acid and tazobactam has almost doubled (Towner *et al.* 1994; Paterson 2006). Moreover, in 2000 the proportion of resistant strains to these two antibiotic combinations did not significantly differ between human and sewage sludge, while in 2009 significantly higher resistances to these combinations were observed in human samples (and amoxicillin/clavulanic resistance in sewage sludge samples was even declining).

Data in this study concerning these antibiotics are also confirmed by other studies, which have found similar resistance rates for piperacillin/tazobactam (0.7%, 0.9%) in wastewater and sewage sludge; for amoxicillin/clavulanic acid, however, higher rates of resistance (7.8%, 9.5%) were found in isolates from the same samples (Holzel *et al.* 2010; Luczkiewicz *et al.* 2010). The only antibiotic with a high proportion of resistances in isolates from both sources was cefalotin in both years.

Considering the relatively similar resistance rates of human and environmental isolates to cefalotin and cefuroxime-axetil (in 2000 as well as 2009), cephalosporin resistance may be more likely to spread effectively into the environment than other resistances. A possible explanation could be reduced competitive fitness of strains carrying trimethoprim or quinolone resistances in comparison to strains carrying cephalosporin resistances. Notably, a high proportion of strains displayed only intermediate resistances to cephalosporin, whereas intermediate resistances to the other antibiotics were rarely found. The maintenance of such intermediate resistances under non-selective conditions is presumably less energy consuming than keeping up high level resistances, hence increasing the chance to endure in a competitive situation.

Further, the persistence of resistances outside health care institutions and the human population does not only depend on the presence of specific antibiotics and resistant strains in the human reservoir. Other factors that have an effect on the survival of resistances in the environment are changes in some genetic characteristics, like the transfer of a genomic resistance mechanism to a mobile genetic element (like transposons with genes for trimethoprim resistance) or co-selection of a resistance with a second antibiotic resistance (Towner *et al.* 1994; Paterson 2006).

Another possible explanation for differences concerning some antibiotics could be the presence of a time lag between the occurrence of antibiotic resistances in humans and their appearance in the environment. We speculate that such a time lag might exist in the case of trimethoprim/sulfamethoxazole or amoxicillin/clavulanic resistance, although more time points would be necessary to confirm this hypothesis. The routes for transmission of resistances from humans into

the sewerage system and to wastewater treatment is short and cannot explain the occurrence of time lags. However, other factors, like genetic changes in the bacteria or other man-made changes, can cause lags (like the use of antibiotics outside medical institutions, for example, in farming) (Carattoli 2008; Deurenberg & Stobberingh 2008; Springer *et al.* 2009).

Future studies will be necessary that include genetic analyses to identify resistance mechanisms of dominant strains in humans and the environment. Furthermore, it will be important to investigate isolates from other sources (like stool samples from healthy humans).

CONCLUSIONS

Our study shows that, for most investigated antibiotic resistances, a parallel development of resistance patterns has taken place over time in both patient and environmental samples. Some antibiotic resistances which were not found in 2000 appeared for the first time in 2009, but also the occurrence of multi-resistant *E. coli* strains in samples of both sources increased over time in both sample types. This matches with the observed worldwide phenomenon of spreading of multi-resistant bacteria out of medical institutions into the environment and consequently into sources of human food supply.

Further surveillance of the process of transmission of antibiotic resistances into the environment is recommended, as well as a consideration of more effective methods to eliminate resistant bacteria and antibiotics in wastewater.

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