Wastewater treatment contributes to selective increase of antibiotic resistance among *Acinetobacter* spp.

Yongli Zhang [a,1], Carl F. Marrs [b], Carl Simon [c], Chuanwu Xi [a,*]

[a] *Department of Environmental Health Sciences, University of Michigan, Ann Arbor, USA*
[b] *Department of Epidemiology, University of Michigan, Ann Arbor, USA*
[c] *Department of Mathematics, University of Michigan, Ann Arbor, USA*

ARTICLE INFO

ABSTRACT

The occurrence and spread of multi-drug resistant bacteria is a pressing public health problem. The emergence of bacterial resistance to antibiotics is common in areas where antibiotics are heavily used, and antibiotic-resistant bacteria also increasingly occur in aquatic environments. The purpose of the present study was to evaluate the impact of the wastewater treatment process on the prevalence of antibiotic resistance in *Acinetobacter* spp. in the wastewater and its receiving water. During two different events (high-temperature, high-flow, 31 °C; and low-temperature, low-flow, 8 °C), 366 strains of *Acinetobacter* spp. were isolated from five different sites, three in a wastewater treatment plant (raw influent, second effluent, and final effluent) and two in the receiving body (upstream and downstream of the treated wastewater discharge point). The antibiotic susceptibility phenotypes were determined by the disc-diffusion method for 8 antibiotics, amoxicillin/clavulanic acid (AMC), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (CL), gentamicin (GM), rifampin (RA), sulfisoxazole (SU), and trimethoprim (TMP). The prevalence of antibiotic resistance in *Acinetobacter* isolates to AMC, CHL, RA, and multi-drug (three antibiotics or more) significantly increased ($p<0.01$) from the raw influent samples (AMC, 8.7%; CHL, 25.2%; RA, 63.1%; multi-drug, 33.0%) to the final effluent samples (AMC, 37.9%; CHL, 69.0%; RA, 84.5%; multi-drug, 72.4%), and was significantly higher ($p<0.05$) in the downstream samples (AMC, 25.8%; CHL, 48.4%; RA, 85.5%; multi-drug, 56.5%) than in the upstream samples (AMC, 9.5%; CHL, 27.0%; RA, 65.1%; multi-drug, 28.6%). These results suggest that wastewater treatment process contributes to the selective increase of antibiotic resistant bacteria and the occurrence of multi-drug resistant bacteria in aquatic environments.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Bacterial resistance to antibiotics limits treatment options, increases morbidity and mortality, and increases the risk of antibiotic-associated adverse events. Resistance is common where antibiotics are heavily used, and additionally antibiotic resistant bacteria are present in wastewater, surface water, ground water, sediments and soils, and increasingly in aquatic environments (Baquero et al., 2008; Klare et al., 1995; Kummerer, 2004; Martinez, 2008; Zhang et al., 2009). Antibiotic use selects for existing resistance mechanisms and for novel resistance mutations (Bywater, 2004, 2005; Finch, 2004; Wassenaar, 2005). Resistance can also be acquired through horizontal gene transfer via uptake of resistance determi-nants via conjugation, transduction and transformation (Davison, 1999; Lorenz and Wackernagel, 1994; Thomas and Nielsen, 2005).

Wastewater treatment plants (WWTP) are important reservoirs of commensal human and animal bacteria in which antibiotic resistant organisms, and/or, determinants persist in the final effluent and are released to the environment (Davison, 1999; Lorenz and Wackernagel, 1994; Thomas and Nielsen, 2005). By linking different environmental compartments including municipal sewage and surface water, WWTP may facilitate spread of antibiotics, antibiotic resistance genes, and antibiotic resistant bacteria between these compartments (Schluter et al., 2007). Furthermore, the high microbial density and diversity of biofilms and activated sludge may facilitate genetic exchange in WWTP (Schluter et al., 2007) and antimicrobial agents are found present wastewater (Kummerer, 2003). These conditions may lead to a selection of antibiotic resistant bacteria in WWTP.

In recent years, many researchers have investigated the effect of the wastewater treatment process on the prevalence of antibiotic resistant bacteria in WWTP or its receiving water body (Goni-Urriza et al., 2000; Guardabassi et al., 1998, 2002; Silva et al., 2006, 2007), but to the best of our knowledge, no studies have investigated the

antibiotic resistant pattern in both the WWTP and its receiving water simultaneously.

Bacteria of the genus *Acinetobacter* are found in many environments, including water, soil, sewage and food. At least 0.001% of the total culturable, heterotrophic, aerobic bacterial population in water and soil is estimated to be *Acinetobacter* spp. (Baumann, 1968; Berlau et al., 1999). *Acinetobacter* spp. have a remarkable ability to develop resistance to antimicrobial agents, and a prodigious capacity to acquire new determinants of resistance, thus making this genus particularly suitable for monitoring antibiotic resistance in the environment (Bergogne-Berezin, 1995; Bergogne-Berezin and Towner, 1996; Guardabassi et al., 1998; Juni, 1978; Towner, 1997). Although there are reports on antibiotic resistance among *Acinetobacter* spp. from aquatic sources, vegetables, sewage, and the hospital environment (Berlau et al., 1999; Dhakephalkar and Chopade, 1994; Guardabassi et al., 1998, 1999; Hujer et al., 2006; Perez et al., 2007), little is known about the antibiotic resistance of *Acinetobacter* spp. in WWTP and their receiving water body.

In our study, we investigated: (1) the effect of wastewater treatment process on the prevalence of antibiotic resistance in *Acinetobacter* spp.; and (2) the possibility of the spread of antibiotic resistance from WWTP to the receiving water body. We also explored the mediating effects of seasonal changes in temperature and river flow rate.

2. Materials and methods

2.1. Study area and sample collection

The study area was the tertiary wastewater treatment plant in Ann Arbor, Michigan, USA and the Huron River receiving the wastewater discharge. The plant was designed in 1995 for a population of 210,700, and the average daily flow is 29.5 MGD. The plant mostly treats domestic wastewater, with only limited amounts of industrial waste; hospital waste comprises a very small percentage of total raw influent and is not pretreated. Agriculture is not extensively practiced in the area. The receiving water has a pH value around 6.8 and BOD_5 value around 5 mg O_2/L.

Liquid treatment consisted of (i) preliminary treatment (screening and grit removal), (ii) primary treatment (gravity sedimentation tanks), (iii) secondary treatment (activated sludge process utilizing anoxic/oxic biological nutrient removal in aeration basins (ferric chloride added as required to polish effluent for soluble phosphorus) followed by secondary sedimentation), (iv) tertiary treatment (rapid sand filtration of secondary effluent to remove particulates) and (v) disinfection (UV light). About 45% of BOD in the raw influent was removed after primary treatment, 95% after secondary treatment, and 99.5% after tertiary treatment.

Samples were collected from three sites in the plant, namely raw influent (RI), secondary effluent (SE), and final effluent (FE) (after UV disinfection and ready for discharge). Samples were also collected from river sites 300 m upstream (U) and 100 m downstream (D) of the wastewater discharge point. There was no other discharge to the river between the two sampling sites.

In order to consider the effect of environmental temperature and river flow, samples were collected on two occasions, April 2007 (low-temperature, low-flow, 8 °C) and August 2007 (high-temperature, high-flow, 31 °C).

At each site, four samples were collected at the same time. Each sample consisted of 1000 mL water collected in a sterile bottle, which was then kept in an ice bath during transportation to the lab, and processed within 12 h of collection.

2.2. Heterotrophic plate counts (HPC)

The heterotrophic bacterial number in the water was determined by heterotrophic plate counts (HPC). A 100 mL aliquot of water from serial ten-fold dilutions for each water sample was filtered through a 0.22 μm-pore membrane (Millipore, Billerica, MA) which was then placed on R_2A agar (Remel, Lenexa, KS) plates. Four replicates were examined for each sample (total 16 replicates). Plates were incubated at 35 °C for 72 h, and then at room temperature for up to one week to ensure the growth of the slow-growing organisms prior to counting colony forming units (CFUs).

2.3. Isolation and identification of Acinetobacter spp.

A 100 mL aliquot of water of serial ten-fold dilutions for each sample was filtered through a 0.22 μm pore membrane (Millipore, Billerica, MA) which was then placed on the *Acinetobacter* spp. selective agar LAM (Jawad et al., 1994). Four replicates were examined for each sample. After incubation at 30 °C for 48 h, countable plates containing at least 20 colonies were used for colony counting and *Acinetobacter* isolation. Around half of the colonies on each plate were picked, and re-streaked onto LB agar (Fisher Sci, Houston, TX) to obtain pure cultures.

Acinetobacter spp. isolates were identified by colony PCR using an *Acinetobacter* spp. specific 16S rRNA primer set Ac436f and Ac676r, the specificity of which has been evaluated by Vanbroekhoven et al. (2004). Single colonies grown overnight were suspended in 100 μL sterile MQ water, and this suspended bacterial solution was used as a PCR template. PCR reactions were carried out in a 25 μL volume reaction, including 1× PCR reaction buffer (Promega, Madison, WI); 0.1 mM dNTPs (Promega Madison, WI), 0.2 μM primers, 1.75 units of Taq DNA polymerase (Promega, Madison, WI), and 1 μL of template. *Acinetobacter* spp. strain Ac811 was used as a positive control. *Acinetobacter* sp. AC811 is a derivative of an environmental isolate of *Acinetobacter* sp. ADP1. The strain belongs to the newly described species *Acinetobacter baylyi* (Vaneechoutte et al., 2006). *Escherichia coli* K12 and all the reagents without any template were used as negative controls.

The amplification program consisted of initial denaturing at 95 °C for 5 min, followed by 30 cycles of 15 s at 95 °C, 30 s at 58 °C, 40 s at 72 °C, and a final extension step at 72 °C for 4 min. Aliquots of 5 μL of each reaction solution were analyzed by electrophoresis on a 2.0% (wt./vol.) agarose gel (Invitrogen, Carlsbad, CA). For those colonies that showed positive results, colony PCR was performed again to verify the results and exclude the false positives. Two PCR amplicons were randomly selected, cloned and sequenced. The sequences were more than 99% homologous to the *Acinetobacter* 16S rRNA gene. The number of positive *Acinetobacter* isolates was recorded for further calculation of *Acinetobacter* spp. number, and its proportion of the total bacteria.

2.4. Antibiotic susceptibility test

Antibiotic susceptibility tests were performed using the disc diffusion method (BD, Franklin Lakes, NJ) according to the recommendations of CLSI (CLSI, 2006); *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. Eight antibiotics were selected as representative of commonly used antibiotic classes (abbreviations and disc concentrations are shown in brackets): amoxicillin/clavulanic acid (AMC, 30 μg), chloramphenicol (CHL, 30 μg), ciprofloxacin (CIP, 5 μg), colistin (CL, 10 μg), gentamicin (GM, 10 μg), rifampin (RA, 5 μg), sulfisoxazole (SU, 250 μg), and trimethoprim (TMP, 5 μg). After 18 h incubation at 30 °C, isolates were defined as resistant when the inhibition zone diameters were less than 18 mm for AMC, 13 mm for CHL, 16 mm for CIP, 8 mm for CL, 13 mm for GM, 17 mm for RA, 13 mm for SU, and 11 mm for TMP. The quality control strains were tested every time, and the variations of inhibition zone diameters among different tests did not exceed 1–2 mm.

2.5. Statistics analysis

The Chi-square test was used to compare the prevalence of antibiotic resistance in *Acinetobacter* isolates among the five sampling site, however in the case where any count was less than 5, Fisher's Exact Test was performed. *p*-values<0.05 were considered to be significant.

Raw influent samples were compared with second effluent and final effluent samples, respectively, and upstream samples was compared with downstream samples. Statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL).

3. Results

3.1. The presence of Acinetobacter spp. in wastewater and its receiving river water

The wastewater treatment process decreased the population of total bacteria (HPC) and *Acinetobacter* spp. on both sampling dates, the numbers being about 3 orders of magnitude lower in the final effluent than in the raw influent (Fig. 1). The final effluent had a significantly lower concentration of total bacteria and *Acinetobacter* spp. than did any of the raw and final effluent samples.

The concentration of total bacteria and *Acinetobacter* spp. was not significantly different between upstream and downstream samples collected at both dates (Fig. 1).

A total of 366 isolates were identified as *Acinetobacter* spp. by colony PCR, including 173 isolates from the low-temperature, low-flow event and 193 isolates from the high-temperature, high-flow event. The percentage of *Acinetobacter* spp. in the total HPC was higher in the wastewater (RI, SE, and FE samples) (0.07%–0.09%) than in the river water (U and D samples) (0.02%–0.05%), and was about two times higher in downstream (0.04%–0.05%) than in upstream samples (0.02%–0.03%).

3.2. Antibiotic resistance of Acinetobacter spp. isolates

When all samples were considered together, the *Acinetobacter* spp. isolates were most resistant to TMP (97%), followed by RA (74%), CHL (40%), AMC (20%), TET (X%), SU (8%), CIP (6%), and GM (2%) (Fig. 2). All *Acinetobacter* isolates were susceptible to CL.

Most isolates were resistant to one or two drugs (98% and 78% respectively); resistance to three (45%) or four (19%) drugs was common, with resistance to TMP, RA, CHL, and AMC tending to go together. Resistance to five (2.5%) or six (1.1%) drugs occurred more
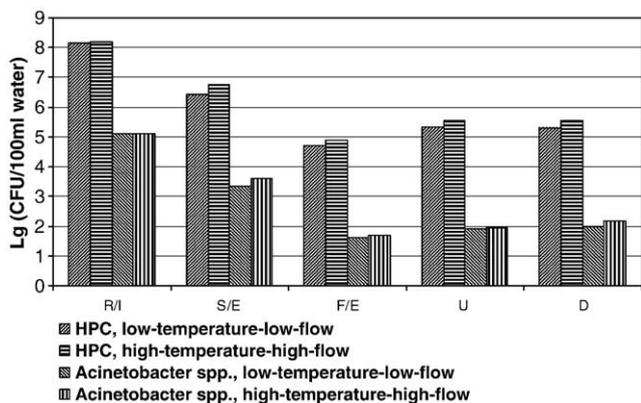
Fig. 1. The concentration of heterotrophic plate counts (HPC) and *Acinetobacter* spp. in water samples from a low-temperature, low-flow and high-temperature, high-flow sampling event. RI, raw influent; SE, second effluent; FE, final effluent; U, upstream; D, downstream.
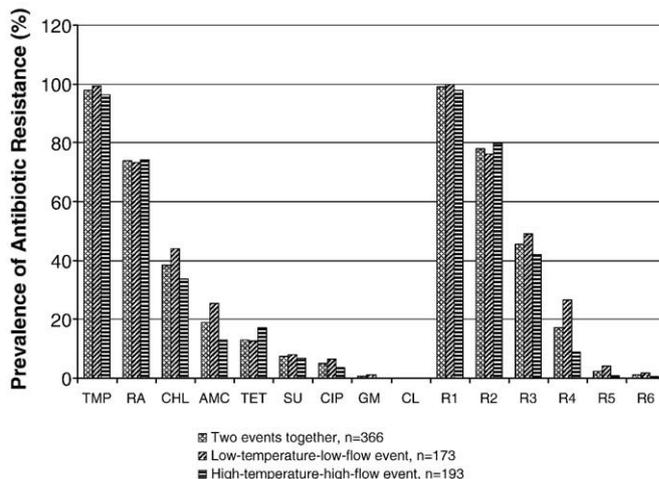
Fig. 2. The prevalence of antibiotic resistance in *Acinetobacter* spp. isolates from all the water samples. *n*, the number of *Acinetobacter* spp. isolates. TMP, trimethoprim, 5 µg; RA, rifampin, 5 µg; CHL, chloramphenicol, 30 µg; AMC, amoxicillin/clavulanic acid, 30 µg; SU, sulfisoxazole, 250 µg; CIP, ciprofloxacin, 5 µg; GM, gentamicin, 10 µg; CL, colistin, 10 µg. R1, R2, R3, R4, R5, R6, resistant to one or more, two or more, three or more, four or more, five or more, six or more antibiotics.

rarely and exclusively in wastewater (RI and SE samples), and was associated with resistance to TMP, RA, CHL, AMC, SU, and CIP.

Similar resistance profiles were observed for both dates; however, the *Acinetobacter* strains isolated from the low-temperature, low-flow event exhibited more resistance than those isolated from the high-temperature, high-flow event (Fig. 2).

The frequency of antibiotic resistance in *Acinetobacter* spp. to RA, CHL, AMC, and multi-drug (three drugs and more) consistently increased from raw influent (RA, 63.1%; CHL, 25.2%; AMC, 8.7%; multi-drug, 33.0%) through second effluent (RA, 77.5%; CHL, 35.0%; AMC, 20.0%; multi-drug, 46.3%) to final effluent (RA, 84.5%; CHL, 69.0%; AMC, 37.9%; multi-drug, 72.4%) (Table 1). The level of resistance to those three antibiotics was significantly higher in the final effluent than in the raw influent (RA, *p*<0.01; CHL, AMC, multi-drug, *p*<0.001). Furthermore, a significantly higher proportion of *Acinetobacter* isolates were resistant to RA (*p*<0.05), AMC (*p*<0.05), SU (*p*<0.01), and R4 (*p*<0.001), R5 (*p*<0.05), and R6 (*p*<0.05) in the second effluent than in the raw influent. *Acinetobacter* isolates resistant to some antibiotics, such as SU, and R5, and R6, were not detected in the final effluent.

Increased resistance was also observed when comparing the sites upstream (AMC, 9.5%; CHL, 27.0%; RA, 65.1%; multi-drug, 28.6%) and downstream (AMC, 25.8%; CHL, 48.4%; RA, 85.5%; multi-drug, 56.5%) of the wastewater discharging point, and the differences were statistically significant (AMC, CHL, *p*<0.05; RA, multi-drug, *p*<0.01).

Resistance to GM, SU, and CIP in *Acinetobacter* spp. was detected exclusively in wastewater (SE; RI and SE; and RI, SE and FE respectively); no *Acinetobacter* strains resistant to these agents were isolated from river water.

The profiles of antibiotic resistance in *Acinetobacter* spp. isolates from different sites were similar at April and August, but the antibiotic resistance level was generally higher at the April low-temperature, low-flow event in comparison with that at the August high-temperature, high-flow event.

4. Discussions

To the best of our knowledge, this is the first study to investigate the impact of the wastewater treatment process on the prevalence of antibiotic resistance in *Acinetobacter* spp. and the possible spread of antibiotic resistance from WWTPs to their receiving water bodies. We

Table 1
The prevalence of antibiotic resistance in *Acinetobacter* spp. isolates from five sampling sites.

Sampling site (n = number of isolates)	TMP	RA	CHL	AMC	SU	CIP	GM	CL	R1	R2	R3	R4	R5	R6
RI (n = 103)	92.2	63.1	25.2	8.7	8.7	4.9	0	0	96.1	66.0	33.0	4.9	1.0	0
SE (n = 80)	100	77.5[a]	35.0	20.0[a]	22.5[a]	11.3	2.5	0	100	81.3[a]	46.3	26.3[a]	10.0[a]	4.5[a]
FE (n = 58)	100	84.5[a]	69.0[a]	37.9[a]	0[a]	6.9	0	0	100	87.9[a]	72.4[a]	37.9[a]	0	0
U (n = 63)	100	65.1	27.0	9.5	0	0	0	0	100	77.8	28.6	6.3	0	0
D (n = 62)	100	85.5[b]	48.4[b]	25.8[b]	0	0	0	0	100	85.5	56.5[b]	17.7[b]	0	0

Prevalence is % of resistant isolates in total isolates.
Note: RI, raw influent; SE, second effluent; FE, final effluent; U, upstream; D, downstream. n, number of isolates. TMP, trimethoprim, 5 µg; RA, rifampin, 5 µg; CHL, chloramphenicol, 30 µg; AMC, amoxicillin/clavulanic acid, 30 µg; SU, sulfisoxazole, 250 µg; CIP, ciprofloxacin, 5 µg; GM, gentamicin, 10 µg; CL, colistin, 10 µg. R1, R2, R3, R4, R5, R6, resistant to one or more, two or more, three or more, four or more, five or more, six or more antibiotics.
[a] Significantly different than raw influent.
[b] Significantly different than upstream.

observed that the prevalence of antibiotic resistance was significantly higher downstream than upstream of the WWTP.

We observed a higher proportion of *Acinetobacter* spp. in wastewater than in river water, suggesting that wastewater effluent could change the distribution of *Acinetobacter* spp. in the receiving water. This is consistent with a previous study which reported that the prevalence of *Acinetobacter* among total cultivable heterotrophic bacteria was higher in samples from downstream than those from upstream of the sewage discharge points of the hospital and the pharmaceutical plant (Guardabassi et al., 1998).

Although we observed a high prevalence of antibiotic resistance in all samples, the isolates from all water samples were generally more susceptible to antibiotics than levels reported for clinical isolates (Bergogne-Berezin and Towner, 1996; Falagas et al., 2007; Henwood et al., 2002), which is consistent with other previous reports (Guardabassi et al., 1998, 1999). Since *Acinetobacter* has a marked ability to develop antibiotic resistance, together with the fact that antibiotics were heavily used in clinical environments, it was not surprising that clinical isolates showed higher level of antibiotic resistance.

Almost all of our isolates were resistant to TMP (97.8%), and most were resistant to RA (73.8%). The high prevalence of resistance to CHL (38.5%) and AMC (18.9%) confirms previous studies which observed that resistance to CHL and AMC were common in *Acinetobacter* spp. (Bergogne-Berezin, 1995; Dhakephalkar and Chopade, 1994; Gerner-Smidt and Frederiksen, 1993). The low prevalence of resistance to SU, CIP, GM, was also in agreement with previous studies (Guardabassi et al., 1999). Isolates resistant to these agents were detected only in wastewater, suggesting that *Acinetobacter* spp. are not inherently resistant to these antibiotics, but probably develop resistance under selective antibiotic pressure or via resistance gene transfer. None of our isolates was resistant to CL, suggesting that CL might still be an effective treatment for infection caused by multi-drug resistant *Acinetobacter* spp. in hospitals (Biancofiore et al., 2007; Pantopoulou et al., 2007).

Previous studies on antibiotic resistant levels in wastewater treatment plants have obtained contradictory results on whether wastewater treatment can increase the prevalence of antibiotic resistant bacteria (Goni-Urriza et al., 2000; Guardabassi et al., 1998, 2002; Silva et al., 2006, 2007), and relatively little has been published on antibiotic resistance in *Acinetobacter* spp. in the wastewater treatment process.

In a study that compared antibiotic resistance among *Acinetobacter* spp. from digested sludge and treated sewage, to isolates from raw water in two tertiary wastewater treatment plants, no significant difference in resistance was observed (Guardabassi et al., 2002). This is in contrast to our results that wastewater treatment was accompanied by a generalized increase in prevalence of antibiotic resistance and multi-drug resistance in *Acinetobacter* spp. isolates, although the population of total bacteria and *Acinetobacter* spp. was effectively reduced in the final effluent (lower than that in the receiving water body). A similar increasing pattern of antibiotic resistance after wastewater treatment has been reported previously,

including resistance to ampicillin (AMP), CHL, and multi-drug in coliforms and resistance to CHL in *Acinetobacter* spp. (Andersen, 1993; Guardabassi et al., 2002; Reinthaler et al., 2003).

We found no previous reports in the literature to support or refute our finding of a higher resistance level in secondary effluent (after activated sludge treatment) compared to raw influent for almost all the tested antibiotics and multi-drug resistance. Our results suggest that the activated sludge process can contribute to a generally selective increase in antibiotic resistance, the acquisition of new antibiotic resistance, and the occurrence of multi-drug resistance in *Acinetobacter* spp. The activated sludge process probably provides a favorable environment with high microbial biomass and selective pressure for horizontal gene transfer of antibiotic resistant genes into *Acinetobacter* spp. cells.

The observed decrease of resistance to some antibiotics in the final effluent could be due to the UV disinfection process after secondary clarification, or to frequencies of resistance to these antibiotics being too low to detect with our sample size. For example, some strains resistant to CIP, SU, GM, R5, and R6 showed poorer growth (data not shown), and may not be easily recovered from the UV treated water.

Although the mechanisms that contribute to selective increase of antibiotic resistant bacteria in WWTP remain undefined, a number of studies have showed that the conditions in WWTP favor proliferation of antibiotic resistant bacteria (Goni-Urriza et al., 2000; Iwane et al., 2001; Schwartz et al., 2003). A few reports revealed that in WWTP, bacteria harbor various plasmids associated with antibiotic resistance genes, which can potentially be transferred to other bacteria and disseminated among bacteria communities (Schluter et al., 2007, 2008). Some studies also indicated that the environmental condition in WWTP may increase the likelihood of gene transfer (Kruse and Sorum, 1994; Mach and Grimes, 1982; Silva et al., 2006). Silva et al. (2006) reported that a high percentage (89%) of multi-resistant coliforms isolated from the sewage treatment plant could partially or completely transfer their resistance patterns to the recipient strain. All these studies suggested that WWTP provides an environment in favor of gene transfer, resulting in the selective increase of antibiotic resistant bacteria in WWTP.

As shown in Table 1, the frequency of antibiotic resistance to RA, CHL, AMC, three or more drugs, and four or more drugs, was higher downstream than upstream in the river receiving wastewater effluent discharges. Guardabassi et al. (1998, 1999) showed the similar results. Furthermore, the pattern of increased antibiotic resistance in riverwater (D) was similar to that in the discharge water (final effluent) from the WWTP, indicating that water discharged from the WWTP may contribute to the dissemination of antibiotic resistance in the receiving water. Thus, it is important to study the fate of multidrug resistant strains in the receiving water body.

A previous study reported that antibiotic and antibiotic resistance gene concentration was lower at higher than lower temperatures during the biological treatment in dairy lagoon (Pei et al., 2007); and a second study reported lower antibiotic concentrations in river sediment during high-flow than low-flow sampling (Pei et al.,

2006). Our results support these observations: *Acinetobacter* spp. strains isolated from the high-temperature, high-flow event were more susceptible to antibiotics than those isolated from low-temperature, low-flow event. High temperature may be more effective for the biodegradation of antibiotics, and high-flow can facilitate the dilution of antibiotics and biomass, which decreases the selective pressure for antibiotic resistance. Therefore, seasonal temperature should be taken into account if biological solids from the wastewater treatment plant are used for land application; similar recommendations have been proposed by others for the use of lagoon water (Peak et al., 2007; Pei et al., 2007).

In conclusion, the biological treatment process in a conventional wastewater treatment plant may result in a selective increase of the antibiotic resistant bacteria population and the increased occurrence of multidrug resistant bacteria. Furthermore, the discharge of wastewater effluent may contribute to the dissemination of antibiotic resistance in the aquatic environment.

References

Andersen SR. Effect of waste water treatment on the species composition and antibiotic resistance of coliform bacteria. Curr Microbiol 1993;26:97-103.

Baquero F, Martinez JL, Canton R. Antibiotics and antibiotic resistance in water environments. Curr Opin Biotechnol 2008;19:260–5.

Baumann P. Isolation of *Acinetobacter* from soil and water. J Bacteriol 1968;96:39–42.

Bergogne-Berezin E. The increasing significance of outbreaks of *Acinetobacter* spp.: the need for control and new agents. J Hosp Infect 1995;30(Suppl):441–52.

Bergogne-Berezin E, Towner KJ. *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. Clin Microbiol Rev 1996;9:148–65.

Berlau J, Aucken HM, Houang E, Pitt TL. Isolation of *Acinetobacter* spp. including *A. baumannii* from vegetables: implications for hospital-acquired infections. J Hosp Infect 1999;42:201–4.

Biancofiore G, Tascini C, Bisa M, Gemignani G, Bindi ML, Leonildi A, et al. Colistin, meropenem and rifampin in a combination therapy for multi-drug-resistant *Acinetobacter baumannii* multifocal infection. A case report. Minerva Anestesiol 2007;73:181–5.

Bywater RJ. Veterinary use of antimicrobials and emergence of resistance in zoonotic and sentinel bacteria in the EU. J Vet Med B Infect Dis Vet Public Health 2004;51:361–3.

Bywater RJ. Identification and surveillance of antimicrobial resistance dissemination in animal production. Poult Sci 2005;84:644–8.

CLSI, Approved standard M2-A9. Performance standards for antimicrobial disk susceptibility tests. Wayne, PA: Clinical and Laboratory Standards Institute (formerly NCCLS), 2006.

Davison J. Genetic exchange between bacteria in the environment. Plasmid 1999;42:73–91.

Dhakephalkar PK, Chopade BA. High levels of multiple metal resistance and its correlation to antibiotic resistance in environmental isolates of *Acinetobacter*. Biometals 1994;7:67–74.

Falagas ME, Mourtzoukou EG, Polemis M, Vatopoulos AC. Trends in antimicrobial resistance of *Acinetobacter baumannii* clinical isolates from hospitalised patients in Greece and treatment implications. Clin Microbiol Infect 2007;13:816–9.

Finch RG. Antibiotic resistance: a view from the prescriber. Nat Rev Microbiol 2004;2:989–94.

Gerner-Smidt P, Frederiksen W. *Acinetobacter* in Denmark: I. Taxonomy, antibiotic susceptibility, and pathogenicity of 112 clinical strains. APMIS 1993;101:815–25.

Goni-Urriza M, Capdepuy M, Arpin C, Raymond N, Caumette P, Quentin C. Impact of an urban effluent on antibiotic resistance of riverine Enterobacteriaceae and *Aeromonas* spp. Appl Environ Microbiol 2000;66:125–32.

Guardabassi L, Dalsgaard A, Olsen JE. Phenotypic characterization and antibiotic resistance of *Acinetobacter* spp. isolated from aquatic sources. J Appl Microbiol 1999;87:659–67.

Guardabassi L, Danilo MA, Wong LF, Dalsgaard A. The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria. Water Res 2002;36:1955–64.

Guardabassi L, Petersen A, Olsen JE, Dalsgaard A. Antibiotic resistance in *Acinetobacter* spp. isolated from sewers receiving waste effluent from a hospital and a pharmaceutical plant. Appl Environ Microbiol 1998;64:3499–502.

Henwood CJ, Gatward T, Warner M, James D, Stockdale MW, Spence RP, et al. Antibiotic resistance among clinical isolates of *Acinetobacter* in the UK, and in vitro evaluation of tigecycline (GAR-936). J Antimicrob Chemother 2002;49:479–87.

Hujer KM, Hujer AM, Hulten EA, Bajaksouzian S, Adams JM, Donskey CJ, et al. Analysis of antibiotic resistance genes in multidrug-resistant *Acinetobacter* sp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center. Antimicrob Agents Chemother 2006;50:4114–23.

Iwane T, Urase T, Yamamoto K. Possible impact of treated wastewater discharge on incidence of antibiotic resistant bacteria in river water. Water Sci Technol 2001;43:91–9.

Jawad A, Hawkey PM, Heritage J, Snelling AM. Description of Leeds *Acinetobacter* Medium, a new selective and differential medium for isolation of clinically important *Acinetobacter* spp., and comparison with Herellea agar and Holton's agar. J Clin Microbiol 1994;32:2353–8.

Juni E. Genetics and physiology of *Acinetobacter*. Annu Rev Microbiol 1978;32:349–71.

Klare I, Heier H, Claus H, Bohme G, Marin S, Seltmann G, et al. *Enterococcus faecium* strains with vanA-mediated high-level glycopeptide resistance isolated from animal foodstuffs and fecal samples of humans in the community. Microb Drug Resist 1995;1:265–72.

Kruse H, Sorum H. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Appl Environ Microbiol 1994;60:4015–21.

Kummerer K. Significance of antibiotics in the environment. J Antimicrob Chemother 2003;52:5–7.

Kummerer K. Resistance in the environment. J Antimicrob Chemother 2004;54:311–20.

Lorenz MG, Wackernagel W. Bacterial gene transfer by natural genetic transformation in the environment. Microbiol Rev 1994;58:563–602.

Mach PA, Grimes DJ. R-plasmid transfer in a wastewater treatment plant. Appl Environ Microbiol 1982;44:1395–403.

Martinez JL. Antibiotics and antibiotic resistance genes in natural environments. Science 2008;321:365–7.

Pantopoulou A, Giamarellos-Bourboulis EJ, Raftogannis M, Tsaganos T, Dontas I, Koutoukas P, et al. Colistin offers prolonged survival in experimental infection by multidrug-resistant *Acinetobacter baumannii*: the significance of co-administration of rifampicin. Int J Antimicrob Agents 2007;29:51–5.

Peak N, Knapp CW, Yang RK, Hanfelt MM, Smith MS, Aga DS, et al. Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. Environ Microbiol 2007;9:143–51.

Pei R, Cha J, Carlson KH, Pruden A. Response of antibiotic resistance genes (ARG) to biological treatment in dairy lagoon water. Environ Sci Technol 2007;41:5108–13.

Pei R, Kim SC, Carlson KH, Pruden A. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). Water Res 2006;40:2427–35.

Perez F, Hujer AM, Hujer KM, Decker BK, Rather PN, Bonomo RA. Global challenge of multidrug-resistant *Acinetobacter baumannii*. Antimicrob Agents Chemother 2007;51:3471–84.

Reinthaler FF, Posch J, Feierl G, Wust G, Haas D, Ruckenbauer G, et al. Antibiotic resistance of *E. coli* in sewage and sludge. Water Res 2003;37:1685–90.

Schluter A, Krause L, Szczepanowski R, Goesmann A, Puhler A. Genetic diversity and composition of a plasmid metagenome from a wastewater treatment plant. J Biotechnol 2008;136:65–76.

Schluter A, Szczepanowski R, Puhler A, Top EM. Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. FEMS Microbiol Rev 2007;31:449–77.

Schwartz T, Kohnen W, Jansen B, Obst U. Detection of antibiotic-resistant bacteria and their resistance genes in waste-water, surface water, and drinking water biofilms. FEMS Microbiol Ecol 2003;43:325–35.

Silva J, Castillo G, Callejas L, Lopez H, Olmos J. Frequency of transferable multiple antibiotic resistance amongst coliform bacteria isolated from a treated sewage effluent in Antofagasta, Chile. Electron J Biotechnol 2006;9:533–40.

Silva MF, Vaz-Moreira L, Gonzalez-Pajuelo M, Nunnes OC, Manaia CM. Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. FEMS Microbiol Ecol 2007;60:166–76.

Thomas CM, Nielsen KM. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat Rev Microbiol 2005;3:711–21.

Towner KJ. Clinical importance and antibiotic resistance of *Acinetobacter* spp. Proceedings of a symposium held on 4–5 November 1996 at Eilat, Israel. J Med Microbiol 1997;46:721–46.

Vanbroekhoven K, Ryngaert A, Wattiau P, Mot RD, Springael D. *Acinetobacter* diversity in environmental samples assessed by 16S rRNA gene PCR-DGGE fingerprinting. FEMS Microbiol Ecol 2004;50:37–50.

Vaneechoutte M, Young DM, Ornston LN, De Baere T, Nemec A, Van Der Reijden T, et al. Naturally transformable *Acinetobacter* sp. strain ADP1 belongs to the newly described species *Acinetobacter baylyi*. Appl Environ Microbiol 2006;72:932–6.

Wassenaar TM. Use of antimicrobial agents in veterinary medicine and implications for human health. Crit Rev Microbiol 2005;31:155–69.

Zhang XX, Zhang T, Fang HH. Antibiotic resistance genes in water environment. Appl Microbiol Biotechnol 2009 Mar;82(3):397–414.