

Surface water isolates of hemolytic and non-hemolytic *Acinetobacter* with multiple drug and heavy metal resistance ability

Sevilay Akbulut, Fadime Yilmaz and Bulent Içgen

ABSTRACT

Acinetobacter in surface waters are a major concern because of their rapid development of resistance to a wide range of antimicrobials and their ability to persist in these waters for a very long time. Four surface water isolates of *Acinetobacter* having both multidrug- and multimetal-resistant ability were isolated and identified through biochemical tests and 16S rDNA sequencing. Based on these analyses, two hemolytic isolates were affiliated with *Acinetobacter haemolyticus* with an accession number of X81662. The other two non-hemolytic isolates were identified as *Acinetobacter johnsonii* and *Acinetobacter calcoaceticus* and affiliated with accession numbers of Z93440 and AJ888983, respectively. The antibiotic and heavy metal resistance profiles of the isolates were determined by using 26 antibiotics and 17 heavy metals. *Acinetobacter* isolates displayed resistance to β -lactams, cephalosporins, aminoglycosides, and sulfonamides. The hemolytic isolates were found to show resistance to higher numbers of heavy metals than the non-hemolytic ones. Due to a possible health risk of these pathogenic bacteria, a need exists for an accurate assessment of their acquired resistance to multiple drugs and metals.

Key words | 16S rDNA, *Acinetobacter* species, multidrug-resistant, multimetal-resistant, river, surface waters

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INTRODUCTION

The control of hospital-acquired infection caused by multiple-resistant Gram-negative bacilli has proved to be a particular problem during the past two decades. Among them, it is now well-recognized that *Acinetobacter* spp. play a significant role in the colonization and infection of patients admitted to hospitals. *Acinetobacter* spp. are a major concern because of their rapid development of resistance to a wide range of antimicrobials, ability to transform rapidly, surviving desiccation, and persistence in the environment for a very long time. The organisms are associated with bacteremia, pulmonary infections, meningitis, diarrhea, and nosocomial infections with mortality rates of 20 to 60%. Transmission is via person-to-person contact, water and food contamination, and contaminated hospital equipment. The increasing virulence and rapid development

of multidrug resistance by these organisms highlights the need to search for alternatives for chemotherapy (Doughari *et al.* 2011). In the past, *Acinetobacter* spp. were considered saprophytes of little clinical significance (Bergogne-Berezin & Towner 1996), but with the introduction of powerful new antibiotics in clinical practice and agriculture and the use of invasive procedures in hospital intensive care units, drug-resistant hospital- and community-acquired *Acinetobacter* infections have emerged with increasing frequency (Doughari *et al.* 2011). *A. baumannii* is an important emerging nosocomial pathogen worldwide, followed by *A. lwoffii* and *A. haemolyticus* (Berlau *et al.* 1999; Jain & Danziger 2004). This organism is a member of a group of phenotypically similar species that are often grouped together in the *A. baumannii-calcoaceticus* complex

(ABC). ABCs have emerged as healthcare-associated pathogens, in part because they are resilient bacteria with a diverse natural habitat. Not only can they survive in moist environments, but they can also survive for weeks on dry surfaces (Jawad *et al.* 1998; Doughari *et al.* 2011). Outbreak investigations have demonstrated that environmental contamination with ABC can be widespread and serve as sources of infection (Scott *et al.* 2007).

The use of drugs in human and animal healthcare has resulted in the widespread development of resistance not only in humans and animals, but also in the environmental reservoir. Data on the prevalence of multidrug- and heavy metal-resistant *Acinetobacter* in surface waters are necessary to estimate the risk of these surface waters to humans. The risk of human exposure to multidrug-resistant bacteria outside a clinical setting is increasing (Ye *et al.* 2010; Hu *et al.* 2011). Antibiotic-resistant bacteria excreted by humans and animals treated with antibiotics end up in the environment, for instance with the discharge of untreated or partially treated sewage or runoff of manure. Thus, the environment can be considered a collecting vessel of antibiotic-resistant bacteria and resistance genes from human and animal origin. Moreover, water and soil may represent selective pressure through heavy metal polluted environments where resistance genes can be transferred among bacteria from different origins, among which are environmental bacterial species (Endo *et al.* 2002). This may result in the creation of novel combinations of bacterial species and specific antibiotic and heavy metal resistance genes (Ozer *et al.* 2013; Aktan *et al.* 2013; Koc *et al.* 2013). People may become exposed to bacteria in surface waters and soils during recreation in contaminated water, when drinking inadequately treated drinking water, water from unprotected sources, or consuming fresh vegetables that have been either irrigated with contaminated surface waters or grown on contaminated soil (Noble *et al.* 2003). The view of the role of introduced antibiotics and heavy metals, antimicrobial resistant bacteria and genes encoding resistance in nature is changing. There is also evidence of a correlation between tolerance to heavy metals and antibiotic resistance (Wilson & Salyers 2003; Aktan *et al.* 2013; Koc *et al.* 2013; Ozer *et al.* 2013). Therefore research into how dispersal of antibiotics and heavy metals affect the bacterial community in nonclinical settings is essential and urgent. The reason for this is that

these settings can be a potential source for dissemination and development of antimicrobial resistance in pathogenic bacteria, which may find their way back into the human population. Therefore, surface waters have been suggested to play a role in the dissemination and development of antibiotic and heavy metal resistance in these bacteria (Reva & Bezuidt 2012). The aim of this study was to characterize river isolates of *Acinetobacter* resistant to multiple drugs and heavy metals. Identification of the isolates was done by using biochemical tests and 16S rDNA sequencing. After determination of multiple antibiotic and metal resistance profiles, the isolates were further characterized in order to find out the locations of the resistance determinants.

MATERIALS AND METHODS

Sample collection, isolation, and purification of *Acinetobacter* species

Water samples were collected along the river Kızıllırmak extending from 39°56' 53.25"N, 33°25' 04.24"E, 699.5 m to 39°23' 53.41"N, 33°25' 18.44"E, 775 m of the city Kırıkkale, Turkey. The samples were put into sterile screw-capped bottles aseptically, kept in an icebox containing ice packs, and taken immediately to the laboratory. A quantity of 1 mL of water from each of the collected samples was dissolved in 9 mL sterile distilled water and serial dilutions were made. Each dilution was plated on Luria Bertani (LB) agar plates by the standard pour plate method. Plates were incubated at 30 °C for 3 days and colonies differing in morphological characteristics were selected. After the growth of different microorganisms on the plates, each bacterial colony on the basis of its morphological characteristics was selected and further purified by repeated streaking on nutrient agar (NA) plates and identified with Gram staining. Each bacterial culture was then inoculated in nutrient broth, incubated and glycerol stocks were made and frozen at -70 °C. For isolation and purification, strains were routinely grown in LB medium at 30 °C. The strains had biochemical properties that define the genus *Acinetobacter* (Bouvet & Grimont 1986), with identification at the genus level confirmed by the transformation assay (Juni 1972). All suspected cultures of *Acinetobacter* species were

subjected to Gram stain and observed under a light microscope for size, shape, and cell arrangements. Presumptive identification was performed with API 20NE strips (Biomérieux, France). Preparation of the strip was done following the standard procedure (Biomérieux, France). *A. calcoaceticus* ATCC 14987 was used as a positive control and *Pseudomonas aeruginosa* ATCC 9027 was used as a negative control. The following tests were used for biotyping as described previously (Gerner-Smidt et al. 1991): liquefaction of gelatin, hemolysis of sheep erythrocytes, production of urease, and growth at 37 and 44 °C in Bacto brain heart infusion broth (Difco). For the catalase test, pure cultures of the isolates were selected using a sterile loop from the agar slant and mixed with a drop of 3% H₂O₂ on a clean glass slide. The catalase-positive coccobacilli were considered as *Acinetobacter*.

Identification of river isolates by 16S rDNA sequence analyses

Confirmation of the taxonomical status of the selected strains was done by molecular methods. Genomic DNA was isolated and analyzed from *Acinetobacter* isolates by the method of Chen & Kuo (1993). Bacterial 16S rDNA was amplified by using the universal bacterial 16S rDNA primers, F (5'-AGAGTTTGATCCTGGCTCAG-3') and R (5'-GGTGTGTTGATTGTTACGACTT-3'). PCR was performed with a 50 µL reaction mixture containing 1 µL (10 ng) of DNA extract as a template, each primer at a concentration of 5 mM, 25 mM MgCl₂ and dNTPs at a concentration of 2 mM, as well as 1.5 U of *Taq* polymerase and buffer used as recommended by the manufacturer (Fermentas, Germany). After the initial denaturation for 5 min at 94 °C, there were 35 cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. PCR was carried out in a gene Piko Thermal Cycler (Thermo Scientific, USA). The obtained PCR products were purified, using the GeneJET™ PCR Purification Kit (Fermentas, Germany), according to the instructions of the manufacturer, and sequenced. The PCR product was sequenced by 3730 × 1 DNA synthesizer (Applied Biosystems, USA). The two 16S rRNA sequences were aligned and compared with other 16S rRNA genes in the GenBank by using the National

Center for Biotechnology Information (NCBI) basic local alignment search tools BLASTn program (Benson & Karsch-Mizrachi 2000). A distance matrix was generated using the Jukes-Cantor corrected distance model. The phylogenetic trees were created using Weighbor (Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction). The 16S rRNA gene sequences have been deposited with GenBank using BankIt submission tool and have been assigned NCBI accession numbers.

Determination of multimetal resistance

To determine multimetal resistance, NA plates supplemented with heavy metal salts were used (Mergeay & Nies 1985). Stocks of the metal salts were prepared in distilled water, sterilized by filter membrane (0.22 µm), and stored at 4 °C. *Acinetobacter* isolates were inoculated in radial streaks on NA media supplemented with increasing concentrations of each heavy metal salts AlCl₃·6H₂O, LiCl, BaCl₂·2H₂O, CrN₃O₉·9H₂O, MnSO₄·H₂O, Pb(NO₃)₂, Co(NO₃)₂·6H₂O, FeCl₃·6H₂O, Hg(NO₃)₂·H₂O, CuSO₄·5H₂O, SnCl₂·2H₂O, NiSO₄·6H₂O, ZnSO₄·7H₂O, K(SbO)₃·C₄H₄O₆·0.5H₂O, Cd(NO₃)₂·4H₂O, Ag(NO₃), and Sr(NO₃)₂ in varying concentrations of 8 to 5,000 µg mL⁻¹. For metal resistance profile, overnight-grown cultures of *Acinetobacter* isolates were inoculated and incubated at 30 °C. The cultures were incubated for 24–48 h, and growth in each concentration was recorded.

Determination of multi-antibiotic resistance

For antibiotic tolerance LB medium supplemented with 1.8% agar, solidified plates were utilized. Overnight-grown cultures of *Acinetobacter* isolates were used for antibiotic resistance or susceptibility. *A. calcoaceticus* ATCC 14987 was used as a reference strain. Disk diffusion method was used to check the resistance or sensitivity of bacterial strains towards given antibiotics (Bauer & Kirby 1966). *Acinetobacter* isolates were incubated for 24–48 h at 30 °C and the zone of inhibition was measured in millimeters. Antibiotics disks used in this study were amikacin (30 µg mL⁻¹), amoxicillin/CA (30 µg mL⁻¹), ampicillin (10 µg mL⁻¹), aztreonam (30 µg mL⁻¹), bacitracin (10 µg mL⁻¹), cefepime (5 µg mL⁻¹), ceftazidime (30 µg mL⁻¹), ciprofloxacin (5 µg mL⁻¹),

chloramphenicol (30 $\mu\text{g mL}^{-1}$), erythromycin (15 $\mu\text{g mL}^{-1}$), gentamicin (10 $\mu\text{g mL}^{-1}$), imipenem (10 $\mu\text{g mL}^{-1}$), netilmicin (30 $\mu\text{g mL}^{-1}$), oxacillin (1 $\mu\text{g mL}^{-1}$), pefloxacin (5 $\mu\text{g mL}^{-1}$), penicillin (10 $\mu\text{g mL}^{-1}$), piperacillin (100 $\mu\text{g mL}^{-1}$), piperacillin/tazobactam (100/10 $\mu\text{g mL}^{-1}$), rifampin (10 $\mu\text{g mL}^{-1}$), sulbactam/cefoperazone (105 $\mu\text{g mL}^{-1}$), tetracycline (30 $\mu\text{g mL}^{-1}$), ticarcillin (75 $\mu\text{g mL}^{-1}$), ticarcillin/CA (75/10 $\mu\text{g mL}^{-1}$), trimeth-sulfa (25 $\mu\text{g mL}^{-1}$), tobramycin (10 $\mu\text{g mL}^{-1}$), and vancomycin (30 $\mu\text{g mL}^{-1}$).

Isolation of plasmid DNA

Plasmid extraction was carried out using the method described by Akinjogunla & Enabulele (2010). Pure isolates were inoculated on MRS broth and incubated. The grown cells were harvested and suspended in 200 μL of solution A (100 mM glucose, 50 mM Tris hydrochloride pH 8, 10 mM ethylenediaminetetraacetic acid (EDTA)) containing 10 mg of lysozyme per mL and 10 $\mu\text{g mL}^{-1}$ mutanolysin and incubated for 30 min at 37 °C in an incubator. 400 μL of freshly prepared 1% sodium dodecyl sulfate in 0.2 N NaOH was added and the samples were mixed by inverting tubes. 300 μL of a 30% potassium acetate solution (pH 4.8) was added and the samples were mixed by vortexing. After incubating on ice for 5 min, the debris was removed by 5 min at 14,000 rpm centrifugation in a centrifuge (model 5415R; Eppendorf). The supernatant was removed and extracted once with a phenol-chloroform mixture (1:1) and precipitated with an equal volume of isopropanol. The plasmid DNA was then dissolved in 100 μL of TE (Tris and EDTA) buffer. Electrophoresis of the DNA was carried out on a 0.8% agarose gel in a 0.5X concentration of Tris-Borate-EDTA (TBE) buffer. Agarose gel was prepared by boiling 0.8 g of agarose powder in 100 mL of 0.5X TBE buffer. After boiling, the solution was allowed to cool and 10 μL of ethidium bromide was added to the cooled agarose solution. This was poured into a casting tray with a comb placed across its rim to form wells. The gel was allowed to set for 30 min and the comb was removed. 20 μL of the plasmid DNA samples were then loaded into the wells after mixing with 2 μL of bromophenol blue. A DNA molecular weight marker was also loaded into one of the wells. The gel was thereafter electrophoresed in a horizontal tank at a constant voltage of 60 V for about 90 min. After

electrophoresis, plasmid DNA bands were viewed by fluorescence of bound ethidium bromide under a short wave ultraviolet light trans illuminator and the photographs were taken using a digital camera. The DNA bands were matched with those for *Agrobacterium tumefaciens* C58C1 and Lambda DNA/*EcoRI* + *HindIII* digest molecular weight markers. The approximate molecular weight of each plasmid was consequently obtained by extrapolation on graphical plots of molecular weight of markers against the distance traveled by the respective band.

Isolation of chromosomal DNA

Total DNA was isolated from all selected *Acinetobacter* strains using a classical protocol for isolation (Chen & Kuo 1993). 1.5 mL of a saturated culture was harvested with centrifugation for 3 min at 12,000 rpm in a centrifuge (model 5415R; Eppendorf). The cell pellet was resuspended and lysed in 200 μL of lysis buffer (40 mM Tris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, 1% sodium dodecyl sulfate (SDS)) by vigorous pipetting. To remove most proteins and cell debris, 66 μL of 5 M NaCl solution was added and mixed well, and then the viscous mixture was centrifuged at 12,000 rpm for 10 min at 4 °C. After transferring the clear supernatant into a new vial, an equal volume of chloroform was added, and the tube was gently inverted at least 50 times until a milky solution was completely formed. Following centrifugation at 12,000 rpm for 3 min, the extracted supernatant was transferred to another vial and the DNA was precipitated with 100% ethyl alcohol, washed twice with 70% ethyl alcohol, dried in speed-vac, and redissolved in 50 μL TE buffer. RNA was removed by adding RNase in the lysis step for 30 min at 37 °C. Chromosomal DNA was electrophoresed and separated on a 1.0% agarose gel. The gel was visualized under UV after staining with ethidium bromide.

Plasmid curing

Plasmid curing was carried out in order to determine the location (plasmid-borne or chromosomal) of the drug resistance marker(s). The curing (elimination) of the resistant plasmids was done using a sub-inhibitory concentration of 0.10 mg mL^{-1} of acridine orange as described by Akinjogunla & Enabulele (2010). *Acinetobacter* isolates were grown

for 24 h at 30 °C in nutrient broth containing 0.10 mg mL⁻¹ acridine orange. After 24 h, the broth was agitated to homogenize the contents and loop-fulls of the broth medium were then sub-cultured onto Mueller-Hinton agar plates and antibiotic sensitivity testing and plasmid analysis were carried out as previously described.

Transformation

Competent cells for transformation were prepared essentially as described by Juni (1972). DNA concentration was determined by absorbance at 260 nm. The saturating concentration of DNA in this transformation system is approximately 10 µg of DNA mL⁻¹. Competent cells (approximately 10⁹ colony-forming units/mL in 0.9 mL of 0.1 M tris hydroxymethyl aminomethane hydrochloride buffer at pH 7.0 containing 0.1 M CaCl₂) were mixed with 0.1 mL of DNA. After 20 min of contact between cells and DNA at 37 °C, the mixture was centrifuged and the cells were suspended in tryptic soy broth (TSB) to the same volume.

RESULTS AND DISCUSSION

Isolation and identification of *Acinetobacter* species

During this study, out of 27 bacterial isolates, only four Gram-negative, non-motile, oxidase-negative, catalase-positive, non-fermentative, and encapsulated coccobacilli were screened as presumptive *Acinetobacter* spp. Of four isolates, the two strains identified as *A. haemolyticus* produced a clear hemolysis on horse blood agar, and hydrolyzed gelatin. These *A. haemolyticus* river isolates were designated as Mn12 and Zn01. The two non-hemolytic isolates were identified as *A. johnsonii* and *A. calcoaceticus* and designated as Sb01 and Fe10, respectively. Analysis of the 16S rDNA sequences with the already available database also confirmed these results. Based on the phylogenetic analysis, the strains Mn12 and Zn01 were affiliated with *A. haemolyticus* with an accession number of X81662. Both *A. haemolyticus* isolates showed 98% 16S rDNA sequence homology with *A. lwoffii* (Figure 1(a)). The two non-hemolytic isolates Sb01 and Fe10 were identified as *A. johnsonii* and *A. calcoaceticus* and affiliated with an

accession number of Z93440 and AJ888983, respectively. The former non-hemolytic isolate showed 96%, and the latter non-hemolytic isolate showed 97% 16S rDNA sequence homology with *A. haemolyticus* (Figures 1(b) and 1(c)). The methods used in this study yielded reliable results with 100% agreement in terms of the correct classification of *Acinetobacter* spp.

Determination of multiple antibiotic and metal resistance profiles

Antibiotic-resistant *Acinetobacter* are a major public health concern since the bacteria can be easily circulated in the environment. In the present study, the antibiotic resistance profiles of *Acinetobacter* isolates showed that river isolates were resistant to multiple antibiotics (Table 1). Resistances to β-lactams, cephalosporins, aminoglycosides, and sulfonamides were highly common. However, of the four isolates, only *A. haemolyticus* Zn01 did not show any resistance to β-lactams and only one of the non-hemolytic isolates *A. calcoaceticus* Fe10 did not display resistance to aminoglycosides. On the other hand, both of these isolates were the only isolates resistant to quinolone-type antibiotic pefloxacin. All of the *Acinetobacter* spp. were found to be resistant to sulfonamide-type antibiotic trimethoprim-sulfamethoxazole. Treatment of *Acinetobacter* infections has conventionally involved the use of β-lactams, aminoglycosides, and quinolones (Bergogne-Berezin & Towner 1996). However, the increased use of these antibiotics has resulted in the widespread emergence of antibiotic-resistant strains (Bergogne-Berezin & Towner 1996). Our study also confirmed the presence of antibiotic resistance in *Acinetobacter* surface water isolates. Carbapenems, a class of β-lactams with a broad spectrum of antibacterial activity, have widely been used as the mainstay for treatment of infections caused by such antibiotic-resistant strains (Dijkshoorn et al. 2007). Unsurprisingly, *Acinetobacter* strains resistant to carbapenems have also rapidly emerged worldwide (Dijkshoorn et al. 2007). However, we did not find any *Acinetobacter* isolates resistant to carbapenems. Different levels and patterns of antimicrobial susceptibilities have been found among different *Acinetobacter* spp., with several studies reporting a higher occurrence of multidrug resistance in *A. baumannii* compared with the non-*A.*

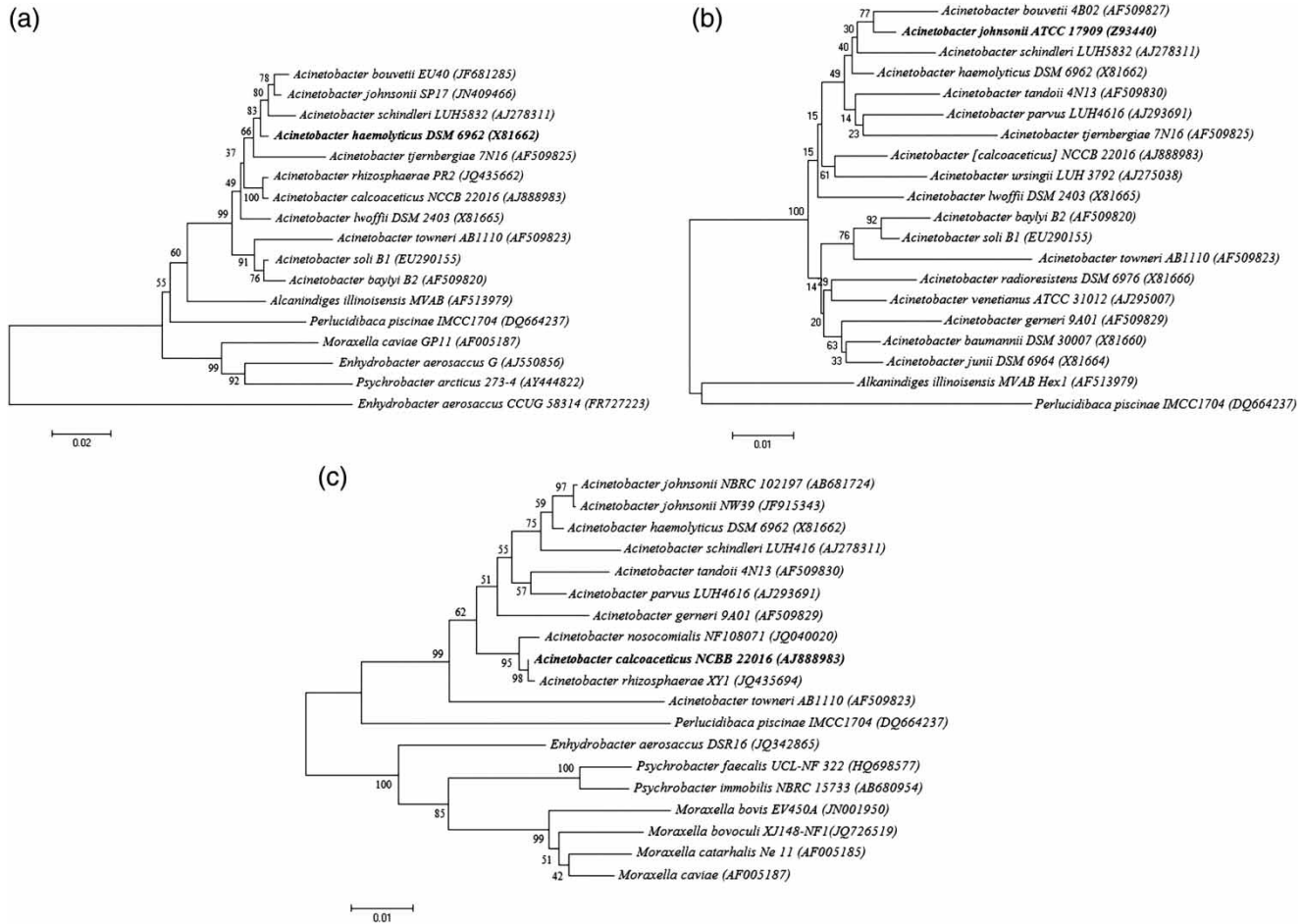


Figure 1 | Phylogenetic trees based on 16S rDNA gene sequence analyses of *A. haemolyticus* isolates Mn12 and Zn01 with an accession number of X81662 (a), *A. johnsonii* isolate Sb01 with an accession number of Z93440 (b), and *A. calcoaceticus* isolate Fe10 with an accession number of AJ888983 (c). The identified isolates and their affiliated numbers are shown in bold. The 16S rDNA sequences are aligned and used to construct the neighbor-joining phylogenetic tree. Scale bar indicates the genetic distance and the numbers shown next to each node indicate the bootstrap values from 1,000 replicons.

baumannii spp. (Van Looveren & Goossens 2004; Turton et al. 2010). Furthermore, intra-species diversity of antimicrobial susceptibilities has also been reported with specific genotypes in the *A. baumannii* population demonstrating higher resistance rates to antimicrobial agents compared with other *A. baumannii* genotypes (Van Looveren & Goossens 2004).

The major problem with *Acinetobacter* spp. is their resistance to antibiotics (Landman et al. 2002). Savov et al. (2002) reported that these organisms are most commonly resistant to ampicillin, cephalothin, carbenicillin, gentamicin, amikacin, chloramphenicol, tetracycline,

co-trimoxazole, ciprofloxacin, and cefoperazone. Previously ampicillin, second-generation cephalosporins, quinolones, minocycline, colistin, aminoglycosides, impenim, sulbactam, and gentamicin were used to treat *Acinetobacter* infections. Resistance to these antibiotics has hindered therapeutic management, causing growing concern throughout the world (Doughari et al. 2011). *A. baumannii* has been developing resistance to all antibiotics used in treating infections. Currently, most *A. baumannii* strains are resistant to aminoglycosides, tetracyclines, cephalosporins, ampicillins, cefotaximes, chloramphenicols, gentamicins, and tobramycins (Prashanth & Badrinath 2005). The activity

Table 1 | Antimicrobial resistance, plasmid and phylogenetic profiles of the *Acinetobacter* river isolates

Strain designation	16S rDNA Identification and (percent homology)	*EMBL Access No.	Plasmid profile (kb)	Metal resistance profile	Antibiotic resistance profile
Mn12	<i>Acinetobacter haemolyticus</i> (99%)	X81662	–	Al, Li, Ba, Pb, Fe, Ag, Cu, Sn, Mn, Ni, Zn, Sb, Sr	ATM, CAZ, GEN, OXA, PIP, SXT, TZP
	<i>Acinetobacter lwoffii</i> (98%)	X81665			
	<i>Acinetobacter schindleri</i> (97%)	AJ278311			
	<i>Acinetobacter soli</i> (96%)	EU290155			
	<i>Acinetobacter baylyi</i> (95%)	AF509820			
	<i>Alkanindiges illinoisensis</i> (94%)	AF513979			
	<i>Acinetobacter towneri</i> (93%)	AF509823			
	<i>Perlucidibaca piscinae</i> (91%)	DQ664237			
	<i>Enhydrobacter aerosaccus</i> (91%)	AJ550856			
	<i>Moraxella caviae</i> (90%)	AF005187			
Zn01	<i>Acinetobacter haemolyticus</i> (99%)	X81662	–	Al, Li, Ba, Mn, Ag, Cu, Sn, Ni, Zn, Sr	GEN, PEF, SXT
	<i>Acinetobacter lwoffii</i> (98%)	X81665			
	<i>Acinetobacter schindleri</i> (97%)	AJ278311			
	<i>Acinetobacter soli</i> (96%)	EU290155			
	<i>Acinetobacter tjernbergiae</i> (95%)	AF509825			
	<i>Alkanindiges illinoisensis</i> (94%)	AF513979			
	<i>Acinetobacter towneri</i> (93%)	AF509823			
	<i>Perlucidibaca piscinae</i> (92%)	DQ664237			
	<i>Enhydrobacter aerosaccus</i> (91%)	AJ550856			
	<i>Moraxella caviae</i> (90%)	AF005187			
Sb01	<i>Acinetobacter johnsonii</i> (97%)	Z93440	17, 42, 117	Li, Ba, Ag, Ni, Sb, Sr	ATM, BAC, OXA, SXT, TIM, TOB
	<i>Acinetobacter haemolyticus</i> (96%)	X81662			
	<i>Acinetobacter gyllenbergii</i> (96%)	AJ293694			
	<i>Acinetobacter lwoffii</i> (96%)	X81665			
	<i>Acinetobacter schindleri</i> (96%)	AJ278311			
Fe10	<i>Acinetobacter calcoaceticus</i> (99%)	AJ888983	–	Fe	CAZ, FEP, OXA, PEF, PEN, PIP, SXT, TZP
	<i>Acinetobacter haemolyticus</i> (98%)	X81662			
	<i>Acinetobacter schindleri</i> (97%)	AJ278311			
	<i>Acinetobacter parvus</i> (96%)	AJ293691			
	<i>Acinetobacter tandoii</i> (95%)	AF509830			
	<i>Acinetobacter generi</i> (94%)	AF509829			
	<i>Acinetobacter towneri</i> (93%)	AF509823			
	<i>Perlucidibaca piscinae</i> (92%)	DQ664237			
	<i>Moraxella catarrhalis</i> (91%)	AF005185			

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Metals: aluminum, Al; antimony, Sb; barium, Ba; Copper, Cu; iron, Fe; silver, Ag; lead, Pb; lithium, Li; manganese, Mn; nickel, Ni; strontium, Sr; tin, Sn; and zinc, Zn.

Antibiotics: aztreonam, ATM; bacitracin, BAC; ceftazidime, CAZ; gentamicin, GEN; oxacillin, OXA; pefloxacin, PEF; penicillin, PEN; piperacillin, PIP; piperacillin-tazobactam, TZP; ticarcillin-clavulanic acid, TIM; tobramycin, TOB; and trimethoprim-sulfamethoxazole, SXT.

of carbapenems is further jeopardized by the emergence of enzymatic and membrane-based mechanisms of resistance (Peleg et al. 2008). Antimicrobial resistance among *Acinetobacter* is either intrinsic or acquired via transformation. Several mechanisms of resistance including altered

penicillin-binding proteins, low/decreased permeability of the outer membrane to antibiotics or increase in the active efflux of the antibiotics, target site mutations, and inactivation via modifying enzymes have been reported (Jain & Danziger 2004). Mechanisms of resistance to antibiotics by

Acinetobacter spp. vary with species, the type of antibiotics, and geographical location (Jain & Danziger 2004). Thus, β -lactam antibiotics are inactivated by the production of β -lactamases or alterations of penicillin-binding proteins and decreased permeability of the outer membrane to β -lactams (Poirel *et al.* 2003); cephalosporins by chromosomally encoded cephalosporinases, and occasionally by cell impermeability and aminoglycosides via aminoglycoside-modifying enzymes; quinolones by altering the target enzymes DNA gyrase and topoisomerase IV through chromosomal mutations, a decrease in permeability and increase in the active efflux of the drug by the microbial cell (Bonomo & Szabo 2006). Treatment of *Acinetobacter* infections should be individualized according to susceptibility patterns as the carbapenems, some fluoroquinolones and doxycycline may retain activity.

Microorganisms resistant to antibiotics and tolerant to metals appear as the result of exposure to metal contaminated environments which cause coincidental co-selection of resistance factors for antibiotics and heavy metals. Heavy metal tolerance in the environment may contribute to the maintenance of antibiotic resistance genes by increasing the selective pressure of the environment. The river isolates of *Acinetobacter* were also tested for their resistance to heavy metals such as Al^{2+} , Pb^{2+} , Li^{2+} , Ba^{2+} , Cr^{3+} , Mn^{2+} , Ag^{2+} , Co^{2+} , Fe^{2+} , Hg^{2+} , Cu^{2+} , Sn^{2+} , Ni^{2+} , Zn^{2+} , Sb^{2+} , Cd^{2+} , and Sr^{2+} at different concentrations from 8 to 5,000 $\mu g mL^{-1}$. The *Acinetobacter* isolates were found to have multiple metal resistance ability (Table 1). Resistance to multiple antibiotics resulted in a general tendency to be resistant to multiple heavy metals except for the non-hemolytic isolate *A. calcoaceticus* Fe10 which only showed single metal resistance to iron. The other non-hemolytic isolate, *A. johnsonii* Sb01, was found to be resistant to heavy metals like silver, lithium, barium, nickel, strontium, and antimony. Almost all *Acinetobacter* isolates were found to be resistant to heavy metals like silver, lithium, barium, nickel, and strontium. Both hemolytic isolates showed resistance to a higher number of heavy metals than the non-hemolytic ones.

Although resistance phenotype determination is of paramount importance for clinical isolates, the tolerance to antimicrobial substances, even when these are below the resistance/susceptibility breakpoints, may represent a selective advantage for the organism in the environment (Faria

et al. 2009). Heavy metals are widespread in sewage as a consequence of industrial pollution and there has been considerable speculation about possible genetic association between bacterial tolerance for these metals and multiple antibiotic resistance (Dhakephalkar & Chopade 1994). The combined resistance to heavy metals was also reported by Silver (1996), Enne *et al.* (2001), Ozer *et al.* (2013), Aktan *et al.* (2013), and Koc *et al.* (2013). Many have speculated and have even shown that a correlation exists between metal tolerance and antibiotic resistance in bacteria because of the likelihood that resistance genes to both antibiotics and heavy metals may be located closely together on the same plasmid in bacteria and are thus more likely to be transferred together in the environment (Endo *et al.* 2002). However, microorganisms may develop resistance at their source, where antibiotic concentrations might be higher, or by acquisition of an antibiotic resistance gene was carried on a genetic element and transferred to that organism via exposure to a different chemical. For example, if a microorganism is in an environment contaminated with heavy metals, the organism may obtain a genetic element from other organisms that survive because they carry a gene responsible for resistance to those metals. If a gene responsible for antibiotic resistance was also present on the same element, it too would be transferred to the organism.

Plasmid DNA profiling, curing, and transformation

It is well known that antimicrobial resistance genes generally reside on extrachromosomal DNA molecule like plasmids. In order to find out the resistance determinants, river isolates of *Acinetobacter* were screened for the presence of plasmid DNA (Table 1). Our study showed that the isolates *A. haemolyticus* Mn12, Zn01, and *A. calcoaceticus* Fe10 did not harbor any plasmid (Figure 2(a)). The multiple drug and heavy metal-resistant genes of these isolates were found to be located on chromosomes. On the other hand, *A. johnsonii* isolate Sb01 contained three plasmids with molecular weights of ca. 17, 42, and 117 kb (Figure 2(b)). Cured derivatives of *A. johnsonii* Sb01 isolate became susceptible to antimony, oxacillin, and bacitracin. The rest of the resistant ability of the cured derivative was found to be retained. In order to confirm these results the isolated plasmids were transformed into antimony, oxacillin,

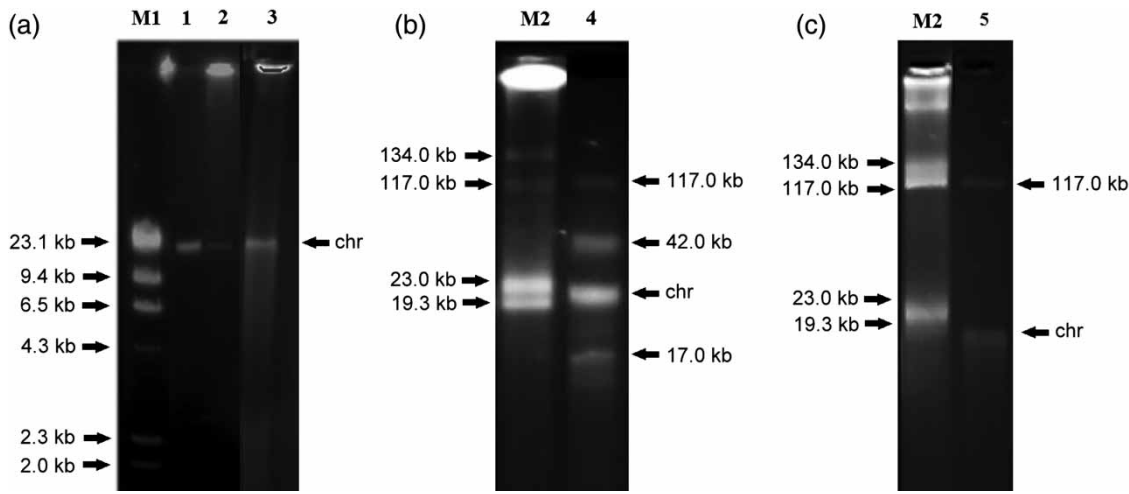


Figure 2 | Plasmid profiles of *A. haemolyticus* isolates Mn12 (lane 1), Zn01 (lane 2), *A. calcoaceticus* isolate Fe10 (lane 3) (a), *A. johnsonii* isolate Sb01 (lane 4) (b), and transformant *E. coli* DH5 α (lane 5) (c). M1, Lambda DNA/EcoRI + HindIII marker; M2, *Agrobacterium tumefaciens* C58C1 marker.

bacitracin-sensitive, plasmid-free and kanamycin-resistant derivatives of *Escherichia coli* DH5 α by the calcium chloride method (Figure 2(c)). The transformation frequency of *E. coli* DH5 α was calculated to be approximately 2.1×10^{-5} per recipient. Transformant *E. coli* DH5 α isolates carrying 117 kb plasmid were able to grow on LB medium containing kanamycin, antimony, oxacillin, and bacitracin. The transformant *E. coli* DH5 α isolates were found to be susceptible to the rest of the antimicrobials used in the study. On the basis of these observations, we concluded that antimony, oxacillin, and bacitracin resistance ability of river isolates of *A. johnsonii* Sb01 was linked and determined by 117 kb plasmid. The rest of the multiple resistance ability of this isolate was thought to be chromosome-encoded.

The success of bacterial pathogens in the environment is driven by their ability to adapt, spread, and establish ecological reservoirs (Hanssen & Ericson Sollid 2006). An important determinant of this adaptation is the acquisition of genes that confer resistance, or increase already existing resistance, to antibiotics and heavy metals (Hanssen & Ericson Sollid 2006; Aktan et al. 2013; Koc et al. 2013; Ozer et al. 2013). Despite the recognized pressures in selecting for antibiotic resistance in environmental pathogens, there is little understanding of the role of the natural environment as a reservoir of *Acinetobacter* and other potentially pathogenic bacteria that might harbor

important antibiotic-resistant genes. Several factors are believed to contribute to the success of plasmid-encoded antibiotic resistance as an evolutionary mechanism. The presence of antibiotic-resistant genes on plasmids, which are normally nonessential for the survival of the organism, provides the bacterial population with a means to reduce the genetic and physiological load on the majority of cells while, through the carriage of plasmids, a minority of cells are able to maintain the genetic diversity of the population (Miranda et al. 2004). Plasmid-borne genes can undergo more radical evolutionary changes without affecting the viability of the cell, as would changes to indispensable chromosomal genes, and established plasmid transfer mechanisms can provide recipient cells with new genetic material which has already been refined by selective pressures elsewhere (Lyon & Skurray 1987). Plasmids can, however, contribute to the development of chromosomal resistance in two ways. First, plasmids, either in part or *in toto*, can integrate into the bacterial chromosome. In the case of plasmids from Gram-negative bacteria, this may involve short segments of DNA, termed insertion sequences (IS), which reside on both plasmid and chromosome and provide limited regions of DNA sequence homology for recombination. Second, plasmids, together with bacteriophages, can act as vectors for transposable DNA elements or transposons. Antimicrobial resistance genes carried by transposons can be translocated

either from one plasmid to another or from a plasmid to a chromosomal site, irrespective of extensive genetic homology. Moreover, events involving transposons can occur independently of the host's general recombination (*rec*) system by a mechanism(s) termed site-specific recombination. Such genes may therefore become established in diverse species in which the vector molecules themselves cannot replicate. In addition to facilitating the spread of antimicrobial resistance, the clustering of transposons carrying different resistance determinants on plasmids, or the chromosome, provides an explanation for the emergence of multi-resistant bacteria (Lyon & Skurray 1987). Heavy metal resistance genes are often found in pathogenicity genomic islands (PAIs) of different highly virulent microorganisms (Kamachi et al. 2006; Levings et al. 2007; Reith et al. 2008; Durante-Mangoni & Zarrilli 2011; Reva & Bezuidt 2012). The distribution of heavy metal resistance operons might be associated with mobile genomic regions. The role of the heavy metal-resistant operons in pathogenicity remains unclear, but their prevalence in PAIs suggests that they may be important factors which pathogens may use for alternative functions such as transport and detoxification of antibiotics and other detrimental compounds. Furthermore, the roles of mercury resistance genes in bacterial resistance toward clinical disinfectants have also been reported (Han et al. 2012). In relation to the latter, an acquired antiseptic and disinfectant resistance of *A. baumannii* was associated with the arsenic and mercury resistance operons (Durante-Mangoni & Zarrilli 2011) which could possibly be of plasmid origin. Plasmids may exchange transposable elements with the chromosomes or sometimes the whole plasmid may be integrated into a bacterial genome. Having been fixed on the chromosome, the mobile element undergoes amelioration, a process that smoothes out differences in oligonucleotide usage patterns of the host chromosome and that of the acquired element (Lawrence & Ochman 1997). These bring new virulence genes or simply activate the gene exchange between potentially pathogenic microorganisms that eventually lead to the achievement of effective combination of pathogenicity determinants in new virulence genomic islands (Bezuidt et al. 2011). This might explain the widespread multiple drug- and heavy metal-resistant *Acinetobacter* isolates in surface waters.

CONCLUSIONS

The presence of antibiotic and heavy metal-resistant bacteria in surface waters used for recreation may pose a health risk. Data on the prevalence of multidrug- and heavy metal-resistant *Acinetobacter* in surface waters are necessary to estimate the risk of these surface waters to humans. Our study revealed two hemolytic and two non-hemolytic surface water isolates. The antibiotic and heavy metal resistance profiles of these isolates displayed resistance to β -lactams, cephalosporins, aminoglycosides, and sulfonamides. Almost all *Acinetobacter* isolates were found to be resistant to heavy metals like silver, lithium, barium, nickel, and strontium. *Acinetobacter* spp. are a major concern because of their rapid development of resistance to a wide range of drugs and heavy metals. The emergence of multidrug and multimetal resistance ability of *Acinetobacter* spp. also plays a crucial role in their *in vitro* and *in vivo* survival. The antimicrobial selective pressure causes conversion of the sensitive species, either by mutation or by induced expression of resistance elements. These bacteria end up in surface water through the discharge of untreated or partially treated sewage mainly derived from industrial factories, healthcare centers, farms, slaughterhouses, and wastewater treatment plants. Importantly, once in the environment, bacteria of different origin come into physical contact and may exchange resistance genes with the endogenous bacterial populations. Despite the generally believed negative impact of acquired resistance on fitness of the bacteria, the multidrug- and multimetal-resistant *Acinetobacter* spp. may remain present in the environment for a long time and pose a health risk, and need to be more accurately assessed.

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