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Diversity and antibiotic resistance of *Acinetobacter* spp. in water from the source to the tap

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Abstract *Acinetobacter* spp. are ubiquitous bacteria in the environment. *Acinetobacter* spp. isolated from a municipal drinking water treatment plant and from connected tap water were identified to the species level on the basis of *rpo*B gene partial sequence analysis. Intraspecies variation was assessed based on the analysis of partial sequences of housekeeping genes (*rpo*B, *gyr*B, and *rec*A). Antibiotic resistance was characterized using the disk diffusion method and isolates were classified as wild or non-wild type (non-WT), according to the observed phenotype. The strains of *Acinetobacter* spp. were related to 11 different validly published species, although three groups of isolates, presenting low *rpo*B sequence similarities with previously described species, may represent new species. Most of the isolates were related to the species *A. johnsonii* and *A. lwoffii*. These two groups, as well as others related to the species *A. parvus* and *A. tjernbergiae*, were detected in the water treatment plant and in tap water. Other strains, related to the species *A. pittii* and *A. beijerinckii*, were isolated only from tap water. Most of the isolates (80 %) demonstrated wild type (WT) to all of the 12 antibiotics tested. Non-WT for tetracycline, meropenem, and ceftazidime, among others, were observed in water treatment plant or in tap water samples. Although, in general, this study suggests a low prevalence of acquired antibiotic resistance in water *Acinetobacter* spp., the potential of some species to acquire and disseminate resistance via drinking water is suggested.

Keywords Drinking water · Antibiotic resistance · ECOFF · *gyr*B · *rec*A · *rpo*B

Introduction

Members of the genus *Acinetobacter* spp. are Gram-negative, obligate aerobic, heterotrophic bacteria with widespread distribution in natural environments (Juni 2005). Given their metabolic versatility and general physiological characteristics, *Acinetobacter* spp. can easily settle in anthropogenic habitats, including wastewater treatment facilities. In these habitats, the roles of *Acinetobacter* spp. on the removal of phosphorus or in the degradation of polycyclic aromatic hydrocarbons are well known (Kim 1997; Thangaraj 2008). However, in these same habitats, *Acinetobacter* spp. may be subjected to selective pressures capable of promoting antibiotic resistance acquisition (Zhang 2009). The tendency noticed worldwide for *Acinetobacter* spp. to develop antibiotic resistance (Henwood 2002; Kuo 2010; Marshall 2007; Tognim 2004; Xu 2008) may, in part, be attributed to the transient colonization of wastewaters and other habitats subject to anthropic pressures.

Acinetobacter spp. are frequently found also in unpolluted sites, such as ground, surface or tap water (Hoefel 2005; Mckeon 1995; Pavlov 2004; WHO 2008). These habitats constitute important sources of bacteria with potential to infect humans, particularly immune-compromised hosts,

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for example, via water baths and room humidifiers (WHO 2008). In this respect, the ability of some *Acinetobacter* spp. to form biofilms is documented to be important (Bhargava 2010; Simões 2010). The recognized status of some *Acinetobacter* spp. as nosocomial agents is, in part, due to the capability to form biofilms (Henwood 2002; Idzenga 2006; Medina 2007; Regalado 2009; Tognim 2004). Although *A. baumannii* is the most frequently reported (Towner 2009), *Acinetobacter* species, such as those of the *A. calcoaceticus–A. baumannii* complex, have been reported also as potentially hazardous for human health. Additionally, members of the species *A. haemolyticus*, *A. lwoffii*, *A. junii*, or *A. radioresistans*, with widespread distribution in the environment, have been referred to as agents of clinical relevance (Dimopoulou 2003; Idzenga 2006; Kappstein 2000; Poirel 2008; Quinteira 2007; Regalado 2009; Spence 2002; Tognim 2004). Given the ubiquity of these species, their capability to cross between different water compartments (waste, surface, and ground), and their capacity to infect humans, the presence of *Acinetobacter* spp. in tap water may represent a potential health risk. In such situations, the severity of the associated risks will depend, among other factors, on the species and the occurrence of acquired antimicrobial resistance. The hypothesis of this work was that drinking water represents a vehicle for *Acinetobacter* spp. dissemination, in which the hazardous potential could be aggravated by reduced antimicrobial susceptibility. To study this hypothesis, the current study was designed to characterize: (i) the diversity of *Acinetobacter* spp. in water sources (drinking water treatment plant and taps) at the species and subspecies levels and (ii) the non-WT susceptibility to antibiotics of different classes.

Materials and methods

Sampling

The bacterial strains examined in this study were isolated from a drinking water treatment plant (WTP) and distribution system, from tap water in 11 houses and in one health care facility (Fig. 1). The houses and health care facility are served by the WTP studied and are located within an area of 270 km^2. Samples from the WTP were collected at eight different locations along the production process: surface water (W1), alluvial wells (W2), after the ozonation step (W3), after the chlorination point (W4; preceded by flocculation and activated carbon filtration), and at four points downstream in the bulk supply distribution system (W5–W8) (Fig. 1). These samples were collected in November 2007 and in September 2009, at sampling points used for routine control of water quality. Tap water samples were collected in April, July, and October 2009 from 11

household taps (T1–T11; from 11 different houses) with low usage (one to four times a month). The tap water from the health care facility (H) was collected in June 2008, from a tap used regularly, located in the sterilization unit. Water samples were collected and processed as described before (Vaz-Moreira 2011c).

Bacterial enumeration and isolation

Cultivable bacterial enumeration and isolation were based on the membrane filtration method, using the culture media, R2A (Difco, Le Pont de Claix, France), *Pseudomonas* Isolation Agar (PIA, Difco), and Tergitol 7-Agar (TTC, Oxoid, Hants, UK) as described before (Vaz-Moreira 2011c). Bacterial isolates for purification were selected after visual examination of culture plates with countable numbers of colonies. The criteria for further isolate purification were: all colonies of morphotypes represented by less than five colonies, half of the colonies of morphotypes represented by five to 10 colonies, and approximately one-third of the colonies of morphotypes represented by more than 10 colonies. Isolates were purified and preserved as described before (Vaz-Moreira 2011c).

### Preliminary identification of presumptive *Acinetobacter* spp.

After the preliminary characterization, as described by Vaz-Moreira et al. (2011c), of a total of 2,833 bacterial isolates, a group of 323 Gram-negative coccobacilli, oxidase-negative, and catalase-positive isolates, assumed presumptively as possible *Acinetobacter* spp., were selected for further analysis. This set of isolates was screened for presence of the *Acinetobacter* spp. 16 S rRNA signature, as described by Vanbroekhoven et al. (2004). PCRs were carried out with the following program: initial denaturation at 95 °C for 5 min; 30 cycles at 95 °C for 15 s, 58 °C for 30 s, 72 °C for 40 s, and a final extension at 72 °C for 4 min. The DNA template of *A. baumannii* CCUG 19096^T, *E. coli* ATCC 25922, and *P. aeruginosa* DSM 1117 were used in each amplification reaction as positive and negative controls.

Identification at the species level and determination of intraspecific variation

Acinetobacter isolate identification was based on analysis of the sequence of the gene for RNA polymerase beta-subunit (*rpo*B) (La Scola 2006). Additionally, two other housekeeping gene sequences, recombinase A (*rec*A) and gyrase beta subunit (*gyr*B), were analyzed. The combined multilocus sequence analysis (MLSA) of the three gene sequences was used to assess the intraspecies genetic variation.

The partial sequences of *rpo*B (902 bp) and *gyr*B (909 bp) were amplified with the primers and conditions

Fig. 1 Schematic representation of the process of drinking water production and distribution analyzed in this study. The locations of water sampling are indicated as *W1–W8* (water treatment plant and distribution system) and *T1–T11* and *H* (*TAP's*)
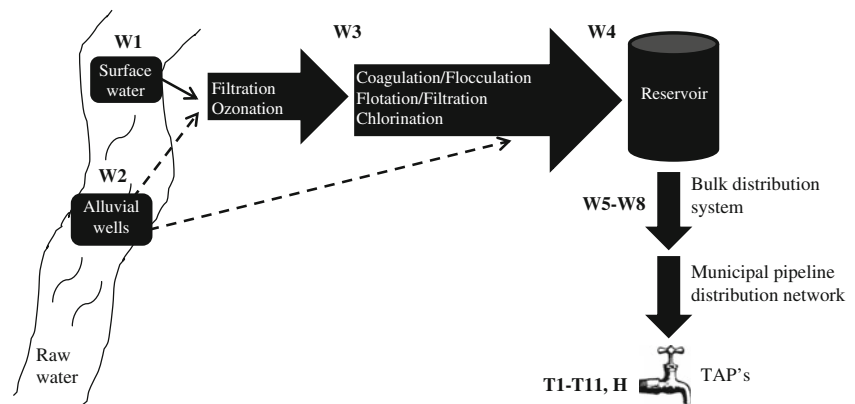
described before (La Scola 2006; Vaz-Moreira 2011b), and *rec*A (425 bp) was amplified with the primers described previously (Nowak and Kur 1995) in reactions of 50 μL, with 1.23 U of Taq PCR Master Mix Kit (Qiagen, Hilden, Germany), 0.5 μM of each primer, RA1 and RA2, and 10 μL of bacterial genomic DNA. After initial denaturation at 95 °C for 2 min, 35 amplification cycles were performed according to the following format: 30 s at 95 °C, 1 min at 55 °C, 2 min at 72 °C, and a final extension of 10 min at 72 °C. PCR products were purified, using the Qiagen DNA Purification Kit (Qiagen, Hilden, Germany), according to the supplier's instructions, and sequenced using a 3130xl Genetic Analyzer (Applied Biosystems, California, USA).

The partial *rpo*B, *rec*A, and *gyr*B nucleotide sequences were edited manually, using the software BioNumerics (ver. 6.1, Applied Maths, Sint-Martens-Latem, Belgium). To determine species affiliations, *rpo*B nucleotide sequences were compared with the *rpo*B sequences of the type strains of all *Acinetobacter* species, available in the GenBank database (http://www.ncbi.nlm.nih.gov) or determined in this study. To assess intraspecies variation, the nucleotide sequences of *rec*A and *gyr*B were also examined. Dendrograms were constructed, based on the model of Jukes and Cantor (1969), using the neighbor-joining, maximum parsimony, and maximum likelihood methods. In the analysis, 828, 852, and 363 nucleotide positions of the *rpo*B, *gyr*B, and *rec*A sequences, respectively, were used. For strain discrimination, the nucleotide sequences of each of the three genes were compared within each species (as determined by *rpo*B sequence analyses). Isolates which sequences differed in, at least, one nucleotide position were considered to represent distinct sequence types (ST).

For simplicity, comparison of the isolates was represented in a dendrogram constructed based on 2,043 positions of the concatenated partial sequences of *rpo*B, *rec*A, and *gyr*B genes (Fig. 2). One representative of each ST of the genes *rpo*B, *rec*A, and *gyr*B was deposited in the GenBank database with the accession numbers (JN903770–JN903895).

Antibiotic resistance phenotypes

Antibiotic resistance phenotypes were determined using the disk diffusion method, according to standard recommendations (Clinical and Laboratory Standards Institute 2007) for all the 244 isolates that gave a positive result for the *Acinetobacter*-specific 16 S rRNA signature. The antibiotics tested were: amoxicillin (AML, 25 μg), gentamicin (GEN, 10 μg), ciprofloxacin (CIP, 5 μg), sulfamethoxazole/trimethoprim (SXT, 23.75/1.25 μg), tetracycline (TET, 30 μg), cephalothin (CP, 30 μg), meropenem (MER, 10 μg), ceftazidime (CEF, 30 μg), ticarcillin (TIC, 75 μg), colistin sulfate (CT, 50 μg), sulfamethoxazole (SUL, 25 μg), and streptomycin (STR, 10 μg) (all Oxoid, Hants, UK). Cultures were incubated for 24 h at 37 °C, except for isolates most related with the species *A. parvus*, which, being unable to grow at that temperature, were incubated at 30 °C. In each experimental set were included the reference strains, *E. coli* ATCC 25922 and *P. aeruginosa* DSM 1117, incubated at temperatures 30 °C and 37 °C. The inhibition zones were measured. For reference strains, an average deviation of 1–2 mm was observed. For each antibiotic, the epidemiological cutoff (ECOFF) value was determined, based on box plot representations of the data for the inhibition diameters observed in this study or, for the antibiotics ciprofloxacin, meropenem, and sulfamethoxazole/trimethoprim, complemented with data available in public databases (http://mic.eucast.org/Eucast2/). Based on box plot representations of the inhibition zone diameters, outliers were identified and classified as non-WT. Thus, isolates with wild type (WT) tolerance to an antibiotic presented inhibition zone diameters which fell inside the box plot bar, whereas non-wild type (non-WT) were outliers.

Statistical analyses

Bacterial richness, diversity, and evenness indices were determined to compare the occurrence of ST of cultivable
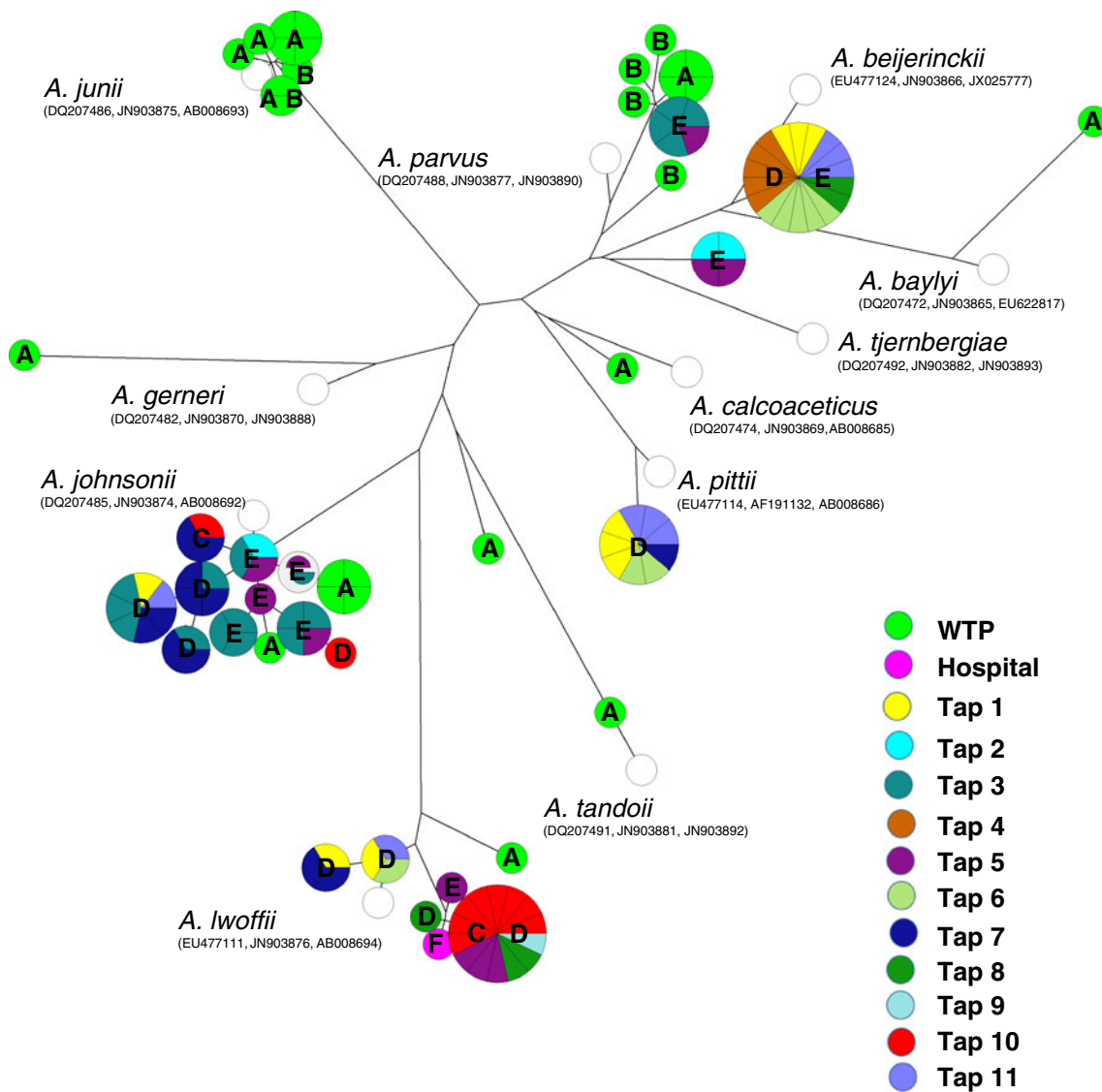
Fig. 2 Maximum likelihood tree of the water isolates and the type strains of *Acinetobacter* spp., constructed on the basis of concatenated *rpo*B, *rec*A, and *gyr*B sequences with the isolation site indicated. Each *circle* represents a different ST. The *circle size* is proportional to the number of isolates with that ST. *White circles* indicate the type strains. The *letters* within the circles refer to the sampling date *A* November 2007, *B* September 2009, *C* April 2009, *D* July 2009, *E* October 2009, *F* June 2008

Acinetobacter spp. over the different sampled sites. The Diversity $[H'=-\Sigma p_i\ln(p_i)]$ and Evenness $[J=H' / \ln(H_{max})]$ were calculated, using the Shannon's (Shannon and Weaver 1949) and Pielou's indices (Pielou 1966), respectively. Species and ST (resultant from the analysis of the three housekeeping genes) were used as the taxonomic units for these calculations, which included all of the isolates ($n=244$), even when recovered on the same date, place, or culture medium. The analyses of the distribution of antibiotic inhibition zones were supported by box plots supplied by SPSS software (version 18.0). The ECOFF values were defined as the values below the lower limits of the normal distribution of inhibition zones.

Results

Diversity of *Acinetobacter* spp.

Cultivable *Acinetobacter* spp. were isolated from samples with total heterotrophic counts ranging from 10^1 to 10^3 CFU mL^{-1} in pretreated surface water in the WTP, 10^{-2}–10^2 CFU mL^{-1} in treated water in the WTP and 10^1–10^4 CFU mL^{-1} in tap water (Table 1). Using PCR- and *Acinetobacter*-specific 16S rRNA gene primers (Vanbroekhoven 2004), 244 isolates out of the 323 presumptive *Acinetobacter* spp. (Gram-negative coco-bacilli, oxidase-negative, and catalase-positive) were indicated to

Table 1 Total heterotrophic counts (CFU mL^{-1}) and percentage (%) of *Acinetobacter* representatives in the different sampled places

Place of isolation		Range of total heterotrophic counts (CFUs mL^{-1})	% of *Acinetobacter* spp. (no. *Acinetobacter*/total no. of isolates)
WTP	W1	$2.5 \times 10^3 - 3.6 \times 10^3$	3.6 % (7/196)
	W2	$1.4 \times 10^1 - 2.1 \times 10^1$	42.7 % (56/131)
	W3	$2.0 \times 10^1 - 1.2 \times 10^3$	1.4 % (2/145)
	W5	$9.7 \times 10^0 - 2.0 \times 10^2$	9.0 % (17/189)
TAP	T1	$2.3 \times 10^2 - 1.6 \times 10^3$	11.1 % (14/126)
	T2	$3.6 \times 10^2 - 1.1 \times 10^3$	3.3 % (4/122)
	T3	$6.0 \times 10^1 - 5.7 \times 10^2$	22.6 % (26/115)
	T4	$2.0 \times 10^2 - 2.0 \times 10^3$	9.0 % (12/133)
	T5	$3.3 \times 10^1 - 3.3 \times 10^2$	16.1 % (24/149)
	T6	$1.5 \times 10^3 - 6.3 \times 10^3$	7.2 % (10/139)
	T7	$2.5 \times 10^2 - 1.8 \times 10^3$	18.7 % (26/139)
	T8	$7.9 \times 10^3 - 7.7 \times 10^4$	5.5 % (8/146)
	T9	$2.9 \times 10^3 - 1.4 \times 10^4$	0.7 % (1/148)
	T10	$2.2 \times 10^3 - 9.9 \times 10^3$	15.4 % (23/149)
	T11	$1.4 \times 10^3 - 1.3 \times 10^4$	6.4 % (8/125)
	H	$3.0 \times 10^1 - 1.3 \times 10^3$	4.3 % (6/140)

Acinetobacter spp. could not be isolated from the WTP sampling point W4 (146 isolates, collected after the chlorination) and in three points of the bulk supply distribution system: W6–W8 (395 isolates)

WTP water treatment plant, *W1* surface water, *W2* alluvial wells, *W3* after ozonation, *W5* before a rechlorination point, *T1–T11* household taps, *H* health care unit tap

be members of this genus. Given the criterion for bacterial isolation (explained in "Materials and methods"), some repetitive isolates could be expected. In order to avoid the artefact of the overrepresentation of some ST or non-wild antibiotic resistance phenotypes, repetitive isolates were removed from further analysis. Thus, when two or more bacterial isolates from the same place, date, and culture medium exhibited identical *rpo*B, *gyr*A, and *rec*A ST and antibiotic resistance patterns, they were considered replicas of the same isolate and were excluded from further analysis. This procedure led to the establishment of a collection of 118 *Acinetobacter* isolates which were compared for their antibiotic resistance types and genotypic diversity. Twenty-four per cent of these isolates (28/118) were from the WTP and 76 % (90/118) were from taps. From the WTP, 22 isolates were collected in November 2007 and six in September 2009. From the taps, five of the isolates were collected in April, 46 in July, 38 in October of 2009, and one in June 2008. None of isolates recovered from three of the four sampling sites located after chlorination points in the WTP (Table 1) were identified as *Acinetobacter* spp.

In an attempt to identify the *Acinetobacter* isolates to the species level, the *rpo*B partial sequences were compared with those of the type strains of all validly published *Acinetobacter* spp. (in January 2012) as recommended by previous studies (La Scola 2006; Gundi 2009). On the basis of analysis of the partial sequence of *rpo*B, the closest neighbors of the *Acinetobacter* isolates were members of 11 distinct species (Table 2). Nevertheless, considering the conclusions of La Scola et al. (2006) that *rpo*B sequence

similarity values below 95 % indicate distinct species, it is hypothesized that seven isolates recovered during this study represent three novel species. The closest neighbors of these seven isolates comprised the species *A. gerneri* (one isolate from W5, 85 % *rpo*B sequence similarity with the type strain), *A. tandoii* (two isolates from W1, 94.2 % *rpo*B sequence similarity with the type strain), and *A. tjernbergiae* (five isolates from W3, T2, and T5, 94.0–94.4 % sequence similarity with the type strain) (Table 2). In general, the analysis of the *gyr*B and *rec*A sequences gave concordant species affiliations with those determined on the basis of *rpo*B nucleotide sequences. Exceptions were observed for strains identified as or most related to *A. baylyi*, *A. gerneri*, *A. parvus*, and *A. tjernbergiae*.

Some lineages were observed in a single type of water or sampling date. For instance, isolates most closely related to the species *A. baylyi*, *A. calcoaceticus*, *A. gerneri*, *A. junii*, and *A. tandoii* were isolated only from WTP samples (Table 2). Others, closely related to *A. pittii* and *A. beijerinckii* were isolated only from tap water. In contrast, isolates related with the species *A. johnsonii*, *A. lwoffii*, *A. parvus*, and *A. tjernbergiae* were obtained from both the water treatment plant and tap water. From alluvial wells, in which human impact is supposed to be lower than in surface water, isolates identified as six different species were observed, *A. baylyi*, *A. calcoaceticus*, *A. junii*, *A. johnsonii*, *A. lwoffii*, and *A. parvus* (Table 2).

In an attempt to determine intraspecies strain diversity and investigate possible clonal relationships between isolates from different sampling sites or dates, concatenated partial nucleotide sequences of the genes *rpo*B, *gyr*B, and

Table 2 Closest neighbors of bacterial isolates based on the *rpo*B sequence analysis, per place of isolation

Closest related species (type strain)	% of *rpo*B sequence similarity (accession number)	Place (number of isolates)
A. baylyi (CIP 107474)	99.4 (DQ207472)	W2 (1)
A. beijerinckii (NIPH 838)	98.3 (EU477124)	Taps (18)
A. calcoaceticus (CIP 81.8)	97.0 (DQ207474)	W5 (1)
A. gerneri (CIP 107464)	85.4[a] (DQ207482)	W2 (1)
A. johnsonii (CIP 64.6)	98.2–99.9 (DQ207485)	W2 (5); taps (31)
A. junii (CIP 64.5)	98.2–98.8 (DQ207486)	W1 (2), W2 (5), W5 (2)
A. lwoffii (NIPH 512)	98.1–99.7 (EU477111)	W2 (1); taps (23)
A. parvus (CIP 108168)	97.8–98.2 (DQ207488)	W1 (3), W2 (3), W5 (1); taps (5)
A. pittii (NIPH 519)	99.7 (EU477114)	Taps (9)
A. tandoii (CIP 107469)	94.2[a]–97.5 (DQ207491)	W1 (1), W3 (1)
A. tjernbergiae (CIP 107465)	94.0–94.4[a] (DQ207492)	W3 (1); taps, (4)

W1 surface water, *W2* alluvial wells, *W3* after ozonation, *W5* downstream from the chlorination tank (see Fig. 1 for details)

[a] *rpo*B sequence similarity values <95 % suggest that these isolates may represent novel species

*rec*A were compared (Table 3, Fig. 2). This analysis supported the definition of 39 ST. Isolates most related with the species *A. pittii*, *A. beijerinckii*, *A. johnsonii*, *A. lwoffii*, and *A. tjernbergiae*, which were represented by more than one isolate and included a single ST, were distributed by different locations and sampling dates. This observation could indicate a possible common origin of these strains (Table 3). In contrast, the occurrence of different lineages was evidenced for isolates which closest neighbors were members of the species *A. johnsonii*, *A. junii*, *A. lwoffii*, *A. parvus*, *A. tandoii*, and *A. tjernbergiae*, represented by up to 13 different ST. Most of these ST were isolated from distinct taps or sampling dates. In other cases, different ST were observed in the same tap (for example, *A. lwoffii* ST lw2, lw4, lw5, lw7, and lw8 in taps 1, 5, 8, and 10; and *A. johnsonii* ST jo2, jo3, jo5, and jo7-14 in taps 3, 5, 7, and 10). This pattern may suggest different episodes of colonization by *Acinetobacter* in the same tap.

No particular ST was ever observed in both water treatment plant and in tap water samples (Fig. 2, Table 3). Similarly, within the water treatment plant, unique ST were detected for each sampled site, with the exceptions of ST ju6 of *A. junii* and ST pa6 of *A. parvus*. Strains with these ST were isolated from alluvial wells and also from samples collected downstream from the chlorination point, hinting at the possible survival of these bacteria during the disinfection process. This can be explained by the fact that, in this treatment facility, water from alluvial wells is not subjected to the treatment stages prior to chlorination, due to its supposed pristine character.

According to the criterion established for bacterial isolation and purification, the set of isolates collected was representative of the variety of cultivable *Acinetobacter* spp. in each location. Based on this assumption, a comparative analysis of the diversity and evenness indices was made. The comparison of the ST diversity and evenness in the different sites did not reveal dramatic differences between

the WTP and tap water samples (Fig. 3). Apparently, ozonation, more than chlorination, imposed a marked reduction on the diversity of ST. In general, the diversity observed was higher in pretreated (W1–W2) and in tap water than immediately after disinfection (W3, W5). Evenness was higher in ozonated water (W3) than in water from alluvial wells (W2) or surface water (W1) and, in general, it was lower in tap water than in the water treatment plant.

In contrast to the general trend in the WTP, the same ST was detected in samples from different taps. For instance, the ST lw8 of the species *A. lwoffii* was detected in tap 10 and in taps 5, 8, 9, and 10 with an interval of 3 months, suggesting a continuous supply of this bacterium in water, or that they live and proliferate in household pipes. The temporal persistence of a specific ST in the same site of isolation could be inferred also from its presence at different sampling dates (Fig. 2). Such persistence could be hypothesized for the isolates most related with the species *A. beijerinckii*, *A. junii*, and *A. lwoffii*. An interesting example was given by isolates of *A. junii*, with the same ST being isolated from alluvial wells almost 2 years apart, suggesting the stability of this habitat.

Antimicrobial susceptibility

The term "epidemiological cutoff" (ECOFF), proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), corresponds to the lower limit of the inhibition zone in a WT population distribution. According to the EUCAST definition, a microorganism is defined as WT if it does not exhibit acquired and mutational resistance mechanisms to a given drug. This definition has no correspondence to the clinical response to antimicrobial treatment and does not vary by changing circumstances. ECOFF values have been published for numerous bacterial groups and antimicrobial agents (http://www.eucast.org) and allow a reliable differentiation between wild bacteria and those

Table 3 Diversity of *Acinetobacter* spp. sequence types according to isolation site and antibiotic resistance type, classified as WT or non-WT

Closest neighbor	Sequence type (n)	Phenotype	Place (n) WTP (28) 0	Taps (90) 1	2	3	4	5	6	7	8	9	10	11	H
A. baylyi	by2 (1)	AML	W2 (1)												
A. beijerinckii	be2 (18)	WT		3				5		5	2			3	
A. gerneri (85 %)	ge2 (1)	CT CEF	W5 (1)												
A. calcoaceticus	ca2 (1)	AML	W2 (1)												
A. johnsonii	jo2 (1)	WT						1							
	jo3 (3)	WT			1	1									
		TET								1					
	jo4 (1)	CT	W2 (1)												
	jo5 (3)	WT				3									
	jo6 (4)	WT	W2 (4)												
	jo7 (1)	TIC				1									
	jo8 (1)	WT						1							
	jo9 (1)	AML MER SXT SUL											1		
	jo10 (4)	WT				3		1							
	jo11 (7)	WT		1		3				2				1	
	jo12 (3)	WT				1				2					
	jo13 (4)	WT				1				3					
	jo14 (3)	WT								2			1		
A. junii	ju2 (1)	WT	W1 (1)												
	ju3 (1)	WT	W5 (1)												
	ju4 (2)	WT	W2 (2)												
	ju5 (1)	WT	W1 (1)												
	ju6 (4)	WT	W2 (3)/W5 (1)												
A. lwoffii	lw2 (1)	WT						1							
	lw3 (1)	WT	W2 (1)												
	lw4 (3)	WT		1						2					
	lw5 (1)	CT CEF									1				
	lw6 (1)	WT													1
	lw7 (3)	WT		1					1					1	
	lw8 (14)	SUL											1		
		WT						3			2	1	7		
A. parvus	pa2 (1)	WT	W1 (1)												
	pa3 (5)	WT					4								
		GEN							1						
	pa4 (1)	WT	W1 (1)												
	pa5 (1)	WT	W1 (1)												
	pa6 (4)	TET	W2 (2) / W5 (1)												
		WT	W2 (1)												
A. pittii	pi2 (9)	STR		3						1	1			1	
		AML STR									1			2	
A. tandoii (94 %)	ta2 (1)	WT	W3 (1)												
	ta3 (1)	WT	W1 (1)												
A. tjernbergiae (94 %)	tj2 (4)	STR			2			2							
	tj3 (1)	WT	W3 (1)												

Site 0 WTP, *1–11* household taps, *H* refers to health care unit tap, *WT* wild type, the indication of antibiotic means that a non-wild phenotype was observed, *AML* amoxicillin, *GEN* gentamicin, *CIP* ciprofloxacin, *SXT* sulfamethoxazole/trimethoprim, *TET* tetracycline, *CP* cephalothin, *MER* meropenem, *CEF* ceftazidime, *TIC* ticarcillin, *CT* colistin sulfate, *SUL* sulfamethoxazole, *STR* streptomycin, *W1* surface water, *W2* alluvial wells, *W3* after ozonation, *W5* before a rechlorination point
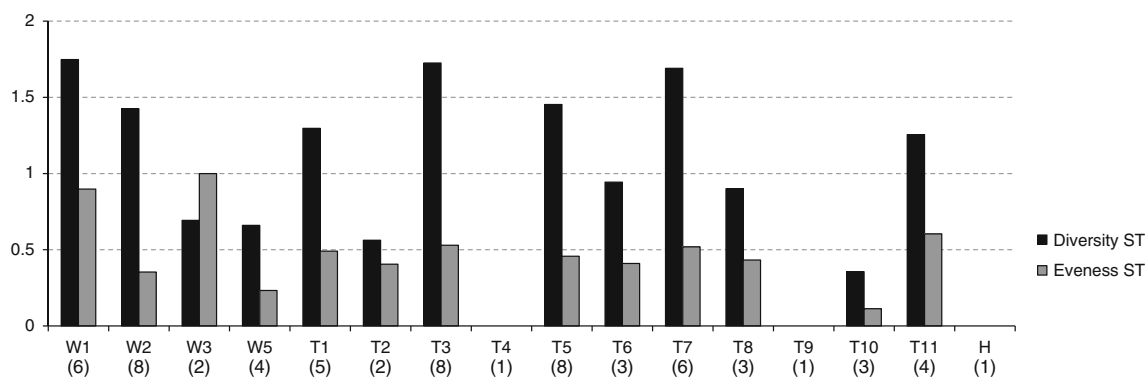
Fig. 3 Diversity and evenness indices, assuming each sequence type as an operational taxonomic unit. The values of richness (number of different sequence types) is indicated below the legend of the sample. Legend details as in Table 3

which acquired any antibiotic resistance mechanism. Nevertheless, for some bacterial groups, ECOFF value databases are still under construction. One of such examples is the genus *Acinetobacter* spp., for which the available data on inhibition zones is scant. Indeed, from the 12 antibiotics used in this study, the ECOFF value was defined only for gentamicin. This fact motivated the calculation of ECOFF values based on the data obtained in the current study, whenever possible, complemented by data available in EUCAST database (Table 4). Isolates were classified as WT or non-WT, although the limited number and origin of strains may have introduced some bias on the data analysis. For instance, in the case of gentamicin, for which an ECOFF value was defined, the value estimated based on our data was ≥18 mm, while EUCAST recommends a value >15 mm. When EUCAST data was included in our dataset, the determined cutoff value for meropenem was lowered from 23 to 20 mm and the value varied for sulfamethoxazole/trimethoprim from 18 to 16 mm. In contrast, for ciprofloxacin, the same value of 20 mm was obtained using

only our dataset or including also the EUCAST inhibition zone data. Cephalothin, for which most of the isolates (>75 %) presented inhibition zones of 0 mm, was excluded from this analysis.

In general, non-WT were rare among the studied *Acinetobacter* spp. from both the WTP and tap water samples, with 80 % of the isolates showing a WT of susceptibility to all the antibiotics tested. Nevertheless, with exception for ciprofloxacin, non-WTs were observed for every antibiotic tested. Non-WT for gentamicin, meropenem, streptomycin, sulfamethoxazole/trimethoprim, sulfamethoxazole, and ticarcillin were detected exclusively among the tap water isolates. In contrast, non-WTs for ceftazidime and colistin sulfate were observed only in WTP. Additionally, non-WTs for tetracycline were significantly more prevalent ($p < 0.05$) among the WTP isolates than in tap water (Table 4). Some non-WTs were observed preferentially in some *Acinetobacter* groups. For instance, non-WTs for streptomycin and tetracycline were restricted to isolates closely related with the species *A. tjernbergiae*

Table 4 Percentage of WTP and tap water isolates with non-wild phenotypes for the different antibiotics tested

Site (n.º isolates)	Epidemiological cutoff values (mm) (n independent determinations)										
	AML <12mm[£] (244)	GEN <15mm* (135)	CIP <20mm[£] (370)	SXT <16mm[£] (370)	TET <18mm[£] (244)	MER <20mm[£] (370)	CEF <14mm[£] (244)	TIC <19mm[£] (244)	CT <13mm[£] (244)	SUL <15mm[£] (244)	STR <15mm[£] (244)
	Percentage of non-WT (%)										
WTP (28)	7.1[9-11]	0	0	0	10.7[10-15]	0	3.6[0]	0	7.1[10-12]	0	0
Tap (90)	4.4[9-11]	1.1[14]	0	1.1[0]	1.1[14]	1.1[17]	0	1.1[12]	0	3.3[0]	14.4[8-11]

Superscripts refer to the range of non-wild inhibition zones values observed (mm)

Shadowed cells correspond to significant differences ($p < 0.05$)

AML amoxicillin, *GEN* gentamicin, *CIP* ciprofloxacin, *SXT* sulfamethoxazole/trimethoprim, *TET* tetracycline, *CP* cephalothin, *MER* meropenem, *CEF* ceftazidime, *TIC* ticarcillin, *CT* colistin sulfate, *SUL* sulfamethoxazole, *STR* streptomycin

[£] Value determined in this study

* EUCAST value

and *A. pittii* or *A. parvus* and *A. johnsonii*, respectively (Table 3). This distribution may suggest the tendency of members of these lineages to acquire those resistance phenotypes.

Discussion

Numerous studies focusing on the diversity of *Acinetobacter* spp., published over the last years, refer to the potential of some members of this genus to act as opportunistic pathogens and develop antibiotic resistance or discuss their role in waste-water biotreatment systems. Although drinking water can harbor different species of *Acinetobacter* (Leclerc and Moreau 2002; Norton and LeChevallier 2000; WHO 2008), to our knowledge, the diversity and dynamics of members of this genus in potable water has not been addressed in the literature. Some studies characterizing the bacterial community through cultivation-independent approaches do not report the detection of *Acinetobacter* spp. in these habitats (Hong 2010; Li 2010; Revetta 2010), while others do confirm the relevance of *Acinetobacter* spp. in potable water (Villarreal, 2010; Vaz-Moreira 2011a; Vaz-Moreira unpublished). In the present work, cultivable *Acinetobacter* spp. were detected in an order of magnitude of 10^4 CFU mL^{-1} of tap water (Table 1), a density which, in some circumstances, may deserve attention.

The selection of the genetic loci used to type *Acinetobacter* spp. was supported by previous publications. The gene *rpo*B, given the low resolution of species detected by the 16S rRNA gene, has become an important and recognized tool for *Acinetobacter* species delineation (La Scola 2006). Species identification, based on the analysis of the partial sequence of the gene *rpo*B, allowed for the differentiation of 11 species groups, three of which include presumably members of novel species. The other two gene sequences analyzed, *gyr*B and *rec*A, constituting part of the recommended protocol for multilocus sequence typing (MLST) of *A. baumannii*, have also been reported to discriminate *Acinetobacter* species (Bartual 2005; Krawczyk 2002; Nowak and Kur 1995; Yamamoto 1999). In this study, the inconsistency of *Acinetobacter* identifications based on the *rpo*B and *gyr*B sequences, previously referred to (La Scola 2006) was confirmed, mainly for strains with the highest *rpo*B sequence similarity to the species *A. baylyi*, *A. gerneri*, and *A. parvus*. Nevertheless, the combination of these three loci (*rpo*B, *gyr*B, and *rec*A) allowed intra-species differentiation for most of the *Acinetobacter* groups analyzed in this work.

Most of the species identified in this study have a recognized environmental distribution. *Acinetobacter* spp. have been isolated from contaminated soils (Vanbroekhoven 2004), freshwater fish (Gonzalez 2000), raw milk (Hantsis-Zacharov and Halpern 2007), or human skin (Seifert 1997).

Isolates most related with the species *A. lwoffii*, *A. johnsonii*, *A. parvus*, and *A. tjernbergiae* were those detected in both the WTP and in tap water. Of the species detected in tap water, *A. lwoffii* and *A. pittii* are those more commonly associated with opportunistic infections (Dimopoulou 2003; Idzenga 2006; Regalado 2009).

Although alluvial wells (W2) were the sampled sites with the highest number of different species (*n*=6), taps 1, 3, 5, 7, and 11 also presented a high diversity of species and/or of ST (Table 3 and Fig. 3). Even when isolates of the same species, i.e., sharing high *rpo*B sequence similarity, were present in both WTP and in tap water, these were always represented by distinct ST (Fig. 2). The occurrence of different strains in distinct types of water suggests the dynamics of *Acinetobacter* populations throughout the water circuit and/or the entrance of *Acinetobacter* spp. strains downstream the water treatment plant. The inclusion of amoebae-resistant *Acinetobacter* spp. by free-living amoebae may be also an important factor influencing the dynamics of members of this genus in disinfected water (Loret and Greub 2010; Thomas 2008, 2010). Nevertheless, a similar distribution of diversity and evenness indices in the water treatment plant and in tap water deny a hypothetical founder effect due to water disinfection (Fig. 3). Further studies in additional water circuits may help to elucidate these hypotheses.

Most of the isolates (80 %) were WT for the antibiotics tested, suggesting low rates of acquired resistance mechanisms. Nevertheless, in both WTP and tap water, isolates with non-WT to different antibiotics were observed. For some antibiotics, non-WT were observed only among tap water isolates, suggesting that tap water *Acinetobacter* spp. do not originate from the WTP or that resistance acquisition to some antibiotics may take place throughout the water distribution. The first hypothesis, which would suggest that *Acinetobacter* spp. detected in tap water enter the system downstream of the WTP, is supported by genotypic and taxonomic analyses. In turn, the fact that tap water isolates with the same ST could be differentiated in WT and non-WT suggests some kind of resistance emergence (mutation or horizontal gene transfer) downstream of the WTP. The relevance of tap water as a potential source of antimicrobial resistant bacteria has been suggested in different studies and bacterial groups (Vaz-Moreira 2012, 2011c ; Xi 2009). Studying cultivable sphingomonads and *Pseudomonas* spp., which are known to occur frequently in drinking water worldwide, Vaz-Moreira et al. (2011c; 2012) showed that these bacteria are relevant potential reservoirs of antibiotic resistance in drinking water. Also Xi et al. (2009) observed the regrowth of bacteria in drinking water distribution systems and concluded that most of the antibiotic resistance genes studied, conferring resistance to beta-lactams or sulfonamides, were more abundant in tap water than in finished disinfected water or source water. Nevertheless, such

differences may be species- or even strain-dependent, as suggested before for sphingomonads and *Pseudomonas* spp. (Vaz-Moreira 2012, 2011c), recovered from the same drinking water system that was analyzed in the current work. The fate of bacteria from the source to the tap, varies according to the taxonomic group, as was demonstrated comparing the present study with another one on aeromonads (Figueira 2011), which in tap water were below the detection limit, presumably due to water disinfection. One of the reasons to explain the capability to colonize tap water can be related with the ability of bacteria to form or adhere to biofilm structures. Such a capacity is demonstrated in *Acinetobacter* spp. (Simões 2010) and may explain the prevalence of these bacteria in tap water and also the liability to acquire antibiotic resistance determinants.

Drinking water quality is influenced by several factors, including geography and climate conditions. Nevertheless, the same bacterial phyla and classes are reported in drinking water worldwide (Eichler 2006; Hoefel 2005; Kormas 2010; Poitelon 2009; Revetta 2010; Thomas 2006; Xi 2009). For this reason, it is expected that studies on bacterial diversity and antibiotic resistance conducted in drinking water treatment plants or tap water worldwide share some general trends. Additionally, these studies are also relevant to allow further inferences on the differences of resistance patterns observed worldwide. *Acinetobacter* spp. are ubiquitous bacteria with the potential to rapidly adapt to the hospital environment and behave as nosocomial pathogens (Gundi 2009; Van Looveren 2004; Visca 2011). The ability to develop antibiotic resistance is part of such an adaptive process and gives some *Acinetobacter* species the character of clinically relevant environmental bacteria (Bergogne-Berezin and Towner 1996; Montealegre 2012). These arguments motivated the current study, conducted in a water supply system feeding about half a million of inhabitants and several health care facilities.

In this study, the highest prevalence of non-WT was observed for the antibiotics amoxicillin, tetracycline, colistin, and streptomycin (Table 4). Some of these resistance phenotypes were previously observed in environmental bacteria of this genus (Dhakephalkar and Chopade 1994). For ceftazidime, tetracycline, and colistin, non-WT were only observed or were more prevalent in the WTP. For instance, for colistin, one of the antibiotics commonly used for the treatment of *Acinetobacter* infections (Fishbain and Peleg 2010), it is a positive finding that no resistance was detected in tap water. It is also noteworthy that isolates related with *A. pittii* were the major harbors of streptomycin non-WT, which was only observed in tap water. Remarkably, *A. pittii* is among the major causes of *Acinetobacter* infection in humans and is a recognized host for new antibiotic resistance determinants (Gundi 2009; Montealegre 2012; Visca 2011).

This study confirms the ubiquity of some species of *Acinetobacter* in water, including in tap water and emphasizes the fact that tap water may represent a vehicle of clinically relevant environmental bacteria to humans. Although multiple sources of colonization, other than the water supply system, may explain the presence of *Acinetobacter* spp. in tap water, their presence and antibiotic resistance patterns deserve attention. Non-WT isolates were rare, but nevertheless, more frequent and diverse in tap water than in WTP, suggesting that the entry of *Acinetobacter* spp. harboring acquired resistance or the acquisition of resistance after water disinfection are likely processes.

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