Universidade do Minho
Escola de Ciências

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Persistence and dispersion of *Acinetobacter* spp. in the urban water cycle

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Professora Doutora Célia Maria Manaia Rodrigues
Professora Doutora Dorit Elisabeth Schuller

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Persistência e dispersão de *Acinetobacter* spp. no ciclo urbano da água

RESUMO

As bactérias do género *Acinetobacter* são ubíquas no solo, esgotos e água. Tendo como objectivo avaliar a persistência e possíveis rotas de dispersão das *Acinetobacter* spp. no ciclo urbano da água, uma colecção de 254 isolados foram recuperados da água de uma estação de tratamento de águas (ETA) e de 12 torneiras. Numa triagem inicial, os isolados foram identificados preliminarmente usando *primers* específicos para 16S ARNr de *Acinetobacter* spp., levando à selecção de 179 isolados. Depois desta triagem, foi usado um esquema de mini-Multilocus Sequencing Typing (MLST) de maneira a conseguir a identificação ao nível da espécie, e quando possível, avaliar padrões de variação intra-específica. Foi determinada a resistência aos antibióticos amoxicilina, ticarcilina, cefalotina, ceftazidime, meropenemo, gentamicina, ciprofloxacina, sulfametoxazol, sulfametoxazol/trimetoprim, tetraciclina, sulfato de colistina e estreptomicina, pelo método de difusão de disco. Os padrões do mini-MLST e os perfis de resistência a antibióticos foram analisados como função do local de isolamento. Visto que não foram observados tipos de sequências (ST) idênticos nos dois ambientes amostrados, concluiu-se que *Acinetobacter* spp. isolados de torneiras não tinham origem na ETA. Não obstante, foi possível inferir a existência de uma fonte comum de *Acinetobacter* spp. nas torneiras, visto ter sido observado o mesmo ST em diferentes torneiras, assim como em distintos sistemas de distribuição de água. A respeito da persistência de *Acinetobacter* spp. nos diferentes locais de amostragem, os isolados com sequências de *rpo*B ou *rec*A idênticas, que foram observadas nos diferentes períodos, foram distinguidos pelo fenótipo de resistência a antibióticos. Além disso, os dados obtidos sugerem que a densidade de *Acinetobacter* spp. no ciclo urbano da água pode aumentar durante o período de Verão, diminuindo durante o Inverno, podendo haver uma nova colonização no ano seguinte. Sete isolados pertencentes ao mesmo grupo de ST, apresentavam distintos perfis de resistência a antibióticos, sugerindo que, ou essas resistências foram adquiridas após a colonização do sistemas de águas, ou o método de tipagem tem baixa resolução. Neste aspecto, admite-se que a análise de sequências nucleotídicas de outros genes contribuirá para uma melhor diferenciação de estirpes e portanto, permitirá ultrapassar a referida limitação.

Persistence and dispersion of *Acinetobacter* spp. in the urban water cycle

ABSTRACT

Members of the genus *Acinetobacter* are ubiquitous in soil, sewage and water. Aiming the assessment of the persistence and possible routes of dispersion of *Acinetobacter* spp. in the urban water cycle, a collection of 254 bacterial isolates were recovered from two different types of water, from a water treatment plant (WTP) and from 12 taps. In the initial screening, isolates were presumptively identified using an *Acinetobacter* spp. specific 16S rRNA primer, leading to the selection of a set of 179 isolates. After this screening, a mini-MLST approach was used in order to achieve the identification to the species level and, whenever possible, to assess patterns of intra-specific variation. The antibiotic resistance to amoxicillin, ticarcillin, cephalothin, ceftazidime, meropenem gentamicin, ciprofloxacin, sulphamethoxazole, sulphamethoxazole/trimethoprim, tetracycline, colistin-sulphate, and streptomycin was determined using the diffusion disc method. The mini-MLST patterns and the antibiotic resistance profile were analyzed as a function of the isolation site. Given that no identical sequencing type (ST) was observed in both types of sampling site, it was concluded that *Acinetobacter* spp. isolated from the taps did not have origin in the WTP. Nevertheless, a common source of tap water *Acinetobacter* spp. was hinted by the observation of the same ST in different taps and water distribution systems (WDS).

In respect to *Acinetobacter* spp. persistence in the different sampling sites, the isolates with identical *rpo*B or *rec*A nucleotide sequences in the different sampling periods, were distinguished by antibiotics resistance patterns. Moreover, it was suggested that the presence of *Acinetobacter* spp. in the urban water cycle rises during the summer period and vanishes during winter, with a new colonization in the following year. Seven isolates belonging to the same ST group, could be distinguished on basis of the antibiogram phenotype, suggesting either that those resistance phenotypes were acquired after the water system colonization, or that the typing method had a the poor resolution . The use of additional gene sequences for strain differentiation will contribute to overcome this limitation.

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INTRODUCTION

The genus *Acinetobacter*

The genus *Acinetobacter* suffered several rearrangements since its description fifty years ago. Originally, the genus *Acinetobacter* included a heterogeneous collection of Gram-negative, oxidase-positive and oxidase-negative saprophytes that could be distinguished from other bacteria by the absence of pigmentation (Ingram & Shewan, 1960). In 1971, after extensive nutritional studies showing differences between the oxidase-negative and the oxidase-positive strains, the Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria suggested that the genus *Acinetobacter* should comprise only oxidase-negative strains. This division was supported by the use of several phenotypic tests, which have now been used for over three decades as the basis for inclusion of individual isolates within the genus (Bergogne-Berezin & Towner, 1996).

Taxonomy and natural habitats

The genus *Acinetobacter*, within the class of *Gammaproteobacteria*, currently comprises 22 validly published species, and more than 10 genomic species (Dijkshoorn *et al.*, 2007, Nemec *et al.*, 2009, Nemec *et al.*, 2010) (Table 1).

Acinetobacters are short, plump, Gram-negative (although sometimes difficult to destain) rods, with cells sizes of about 1.0 to 1.5 by 1.5 to 2.5 μm, in the logarithmic phase of growth, often becoming more coccoid in the stationary phase (Fig. 1). Frequently, within a single pure culture, can be witnessed pairing or clustering of cells, Gram stain variability, as well as variations in cell size and arrangement (Baumann *et al.*, 1968). *Acinetobacter* spp. normally form smooth, sometimes mucoid, pale yellow to grayish-white colonies on solid media, although some environmental strains able to produce a diffusible brown pigment have been described (Pagel & Seyfried, 1976). All members of the genus are strict aerobes, oxidase-negative, catalase-positive, and non-fermentative. A rapid test useful to distinguish *Acinetobacter* spp. from other similar non-fermentative bacteria consists on the detection of cytochrome C oxidase activity. Although nitrate reduction may be undertaken, most strains are unable to reduce nitrate to

Table 1. *Acinetobacter* type strain species (Euzéby, 1998)

Species	Type Strain Number
Acinetobacter baumannii	CCUG 19096^T
Acinetobacter baylyi	CCUG 50765^T
Acinetobacter beijerinckii	CCUG 51249^T
Acinetobacter bereziniae	CCUG 26493^T
Acinetobacter bouvetii	CCUG 50766^T
Acinetobacter calcoaceticus	CCUG 12804^T
Acinetobacter gerneri	CCUG 56316^T
Acinetobacter guillouiae	CCUG 2491^T
Acinetobacter gyllenbergii	CCUG 51248^T
Acinetobacter haemolyticus	CCUG 888^T
Acinetobacter johnsonii	CCUG 19095^T
Acinetobacter junii	CCUG 889^T
Acinetobacter lwoffii	CCUG 33984^T
Acinetobacter parvus	CCUG 48800^T
Acinetobacter radioresistens	CCUG 56440^T
Acinetobacter schindleri	CCUG 45560^T
Acinetobacter soli	CCUG 59023^T
Acinetobacter tandoii	CCUG 56317^T
Acinetobacter tjernbergiae	CCUG 50768^T
Acinetobacter towneri	CCUG 50769^T
Acinetobacter ursingii	CCUG 45559^T
Acinetobacter venetianus	CCUG 45561^T

nitrite in the conventional nitrate reduction assay (Bergogne-Berezin & Towner, 1996). The majority of the strains can grow in a simple mineral medium containing a single carbon and energy source. A wide variety of organic compounds can be used as carbon sources (Baumann*, et al.*, 1968). No single metabolic test enables unambiguous differentiation of this genus from other similar bacteria (Bergogne-Berezin & Towner, 1996). Bacteria of the genus *Acinetobacter* are found in many environments, including water, soil, sewage and food products. At least 0.001% of the total culturable, heterotrophic, aerobic bacterial population in water and soil is estimated to be *Acinetobacter* spp. (Baumann, 1968).

Among the microorganisms participating in activated sludge communities, members of the genus *Acinetobacter* have been attracting increasing attention from a biotechnological point of view, because bacteria of this genus are known to be involved in the biodegradation of a number of different pollutants such as biphenyl (BP) and chlorinated BPs (Furukawa & Chakrabarty, 1982, Shields *et al.*, 1985), aniline (Wyndham, 1986), phenol (Hoyle *et al.*, 1995), crude oil (Amund & Higgins, 1985, Di Cello *et al.*, 1997) and a synthetic lubricant (Amund, 1996).

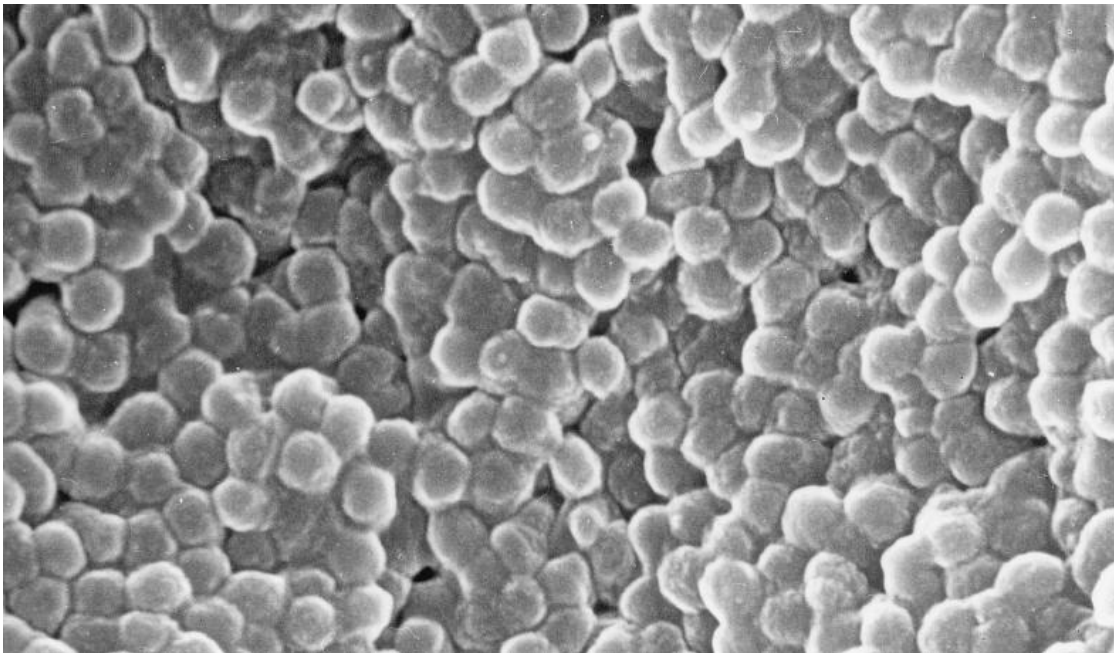

Figure 1. Scanning electron micrograph of *A. baumannii* type strain ATCC 19606 (final magnification, 318,000). Prepared and photographed by A. Shelton. (Bergogne-Berezin & Towner, 1996).

A striking property of these bacteria is their ability to grow at the expenses of a great variety of organic compounds as sole sources of carbon and of nitrate as sole source of nitrogen (Baumann, 1968). Such a metabolic versatility may explain the widespread distribution in the environment. For example, LaCroix & Cabelli (1982) enumerated acinetobacters in surface and in treated drinking water and Bifulco *et al.* (1989) reported its presence in untreated rural groundwater supplies. Besides, *Acinetobacter* spp. are normal inhabitants of the skin in healthy people (Bergogne-Berezin & Towner, 1996). Given this ubiquity, *Acinetobacter* spp. are considered opportunistic pathogens with potential public health significance. In the last three decades, *Acinetobacter* spp. have increased considerably in importance as opportunist human pathogens, being isolated from various types of nosocomial infections, including septicemia, pneumonia, endocarditis, meningitis, skin and wound infection, and urinary tract infection (Bergogne-Berezin & Towner, 1996, Guardabassi *et al.*, 2000). Air contamination in the absence of a colonized patient is rare (Bergogne-Berezin & Towner, 1996), but several studies have documented extensive contamination by *Acinetobacter* spp. of the environment, including respirators and air samples, in the vicinity of infected or colonized patients (Cunha *et al.*, 1980). Another relevant feature of *Acinetobacter* spp. is the ability to rapidly develop antibiotic resistance, perhaps as a consequence of its long-term evolutionary exposure to antibiotic-producing organisms in a soil environment. Some studies have used this genus as a tool for

monitoring antibiotic resistance in the environment, given the prodigious capacity of its members to acquire new determinants of resistance (Bergogne-Berezin, 1995, Bergogne-Berezin & Towner, 1996, Guardabassi *et al.*, 1998).

Identification and typing at the species and strain level

As referred above, the identification of *Acinetobacter* spp. is not a straightforward process, and when based on phenotypic traits it may involve a diversity of tests. In fact, this is a serious limitation in the routine bacteriology of several different groups of bacteria. In the case of organisms of environmental relevance, the identification to the species level is very often of limited value and both, the distinction of individual strains and of their phylogenetic relationship, becomes a priority. In this respect, a panoply of molecular typing methods can offer interesting insights into the ecology and epidemiology of bacteria.

The attention recently given to *Acinetobacter* spp. as opportunistic pathogen has driven the development of reliable methods for phylogenetic analysis and species identification. The widely adopted biochemical scheme for identification of *Acinetobacter* species (Bouvet & Grimont, 1986) showed promising results, but further studies have evidenced that this scheme does not cover the phenotypic variability of all genomic species (Gerner-Smidt *et al.*, 1991).

Due to the widely use of 16S rRNA gene sequence analysis, the comparison of amplified sequences with others available in public databases facilitates the identification of bacterial isolates and allows the inference of the phylogenetic classification. In the case of the genus *Acinetobacter*, the extremely slow rate of the base substitutions in 16S rRNA gene makes the resolution of this phylogenetic marker insufficient to distinguish closely related species (Berlau *et al.*, 1999, Chu *et al.*, 1999).

Thus, as in many other genera, *Acinetobacter* species identification often requires the use of standard methodologies such as DNA-DNA hybridization (Stackebrandt & Goebel, 1994). DNA-DNA hybridization is a time consuming and laborious procedure, unsuitable for routine examinations or to compare large sets of isolates (Tjernberg & Ursing, 1989). Moreover, such methodological approach does not give information about sub-species differentiation as is

desirable in epidemiologic surveys. These difficulties created the need for alternative methods that can be applied to large numbers of strains for epidemiological studies (Krawczyk *et al.*, 2002).

Among the molecular fingerprinting methods available, the pulsed-field gel electrophoresis (PFGE) has been regarded as a relevant methodology, given its reliability, resolution at the strain level, and inter-laboratorial reproducibility (Tenover *et al.*, 1995). Many studies have suggested that PFGE is the most discriminatory technique for characterization of *Acinetobacter* strains (Marcos *et al.*, 1995, Quelle & Catalano, 2001, Seifert & Gerner-Smidt, 1995).This method involves the use of rare-cutter restriction enzymes to generate a limited number (10 to 20) of high-molecular-weight restriction fragments, which are separated in an agarose gel electrophoresis. The use of programmed variations in two perpendicular directions and the duration of the electric field (the pulsed field) allows the separation of the high molecular weight DNA fragments, rendering a profile characteristic of a species or strain. In spite of the above mentioned advantages, PFGE protocols involve time-consuming, tedious procedures for the purification of intact genomic DNA trapped in agarose, lengthy restriction enzyme digests, and extended electrophoresis times (Maslow *et al.*, 1993).

Another identification technique with discriminating power at the strain level, as PFGE, and with the advantage of offering additional phylogenetic insights is the Multilocus Sequence Typing (MLST). MLST is based on the well-tested principles of MLEE that analyses the electrophoretic mobilities of housekeeping enzymes on starch gels and equates the different charge variants of each enzyme with alleles at the underlying genetic locus. It is built an electrophoretic type by the alleles at each locus, but only a few variants are detected at each locus so it is better to analyze twenty or more loci, in order to obtain a good level of resolution. These data can be visualized through a dendrogram obtained from the matrix of pair-wise differences between the electrophoretic types (Selander *et al.*, 1986). The difference observed in MLST is that instead of assigning the alleles at each locus directly from the electrophoretic mobility of the gene product on gels, the assignment is done directly by nucleotide sequencing (Maiden *et al.*, 1998). As can be expected and as confirmed by Enright & Spratt (1999), in MLST sequences of 450–500-bp internal fragments of only seven housekeeping genes are enough to offer sufficient variations to identify many different alleles within a bacterial population. This happens because the

nucleotide sequence exposes all the variation at a locus and the number of alleles assigned per locus is much higher than in MLEE. (Enright & Spratt, 1999) also demonstrated that this length of DNA fragment can be sequenced accurately on both strands using a single pair of primers. For each gene, the different sequences are assigned as alleles and the alleles at the seven loci provide an allelic profile, which unambiguously defines the sequence type (ST) of each isolate. Sequences that differ at even a single nucleotide are assigned as different alleles and no weighting is given to take into account the number of nucleotide differences between alleles, so it is impossible to distinguish whether differences at multiple nucleotide sites are a result of multiple point mutations or a single recombinational exchange (Spratt, 1999). MLST is an ideal method for global epidemiology (Enright & Spratt, 1999) because the accumulation of nucleotide changes in housekeeping genes is a relative slow process and the allelic profile of a bacterial isolate is sufficiently stable over time, even MLST being a highly discriminatory technique. A major advantage of MLST compared to other typing methods results from the use of nucleotide sequence data that offer the possibility of data exchange via an electronic platform. This feature makes this technique ideally suited for national or international surveillance programs involving multiple laboratories and for monitoring the spread of drug-resistant clones (Bartual *et al.*, 2005, Dingle *et al.*, 2001, Enright *et al.*, 2000, Park *et al.*, 2009). It was already demonstrated a MLST scheme for *A. baumannii* (Bartual*, et al.*, 2005, Park*, et al.*, 2009, Wisplinghoff *et al.*, 2008) and genomic species 13TU (Park*, et al.*, 2009, Wisplinghoff*, et al.*, 2008) that provided a powerful tool for molecular epidemiologic studies, and offered a new way to study the population biology of this pathogen. MLST typing system provides a portable method that is suitable for global epidemiologic studies and allows the recognition of epidemic, multiresistant, and virulent clones and monitoring of their international spread. In spite of these, the usefulness of such a method still has to be tested with large numbers of strains (Bartual*, et al.*, 2005, Park*, et al.*, 2009, Wisplinghoff*, et al.*, 2008).

Strains of different species will almost invariably have different alleles at all seven loci, and therefore, the concatenated sequences of the seven loci are used to explore the relationships among the strains of similar species. This extension of the MLST approach has been termed MLSA (Gevers *et al.*, 2005). MLSA compares the primary DNA sequences from multiple conserved protein-coding loci for assessing the diversity and relationship of different isolates across related taxa, thereby using an appropriate phylogenetic or cladistic approach (Naser *et*

al., 2005). The MLSA approach has increasingly been used to establish the phylogenetic position of new species (Christensen *et al.*, 2004) and the relationships between species in closely related genera (Wertz *et al.*, 2003). A few studies have used this approach to look at the relationships among the closely related species within a single genus. For instance, MLSA has been used to resolve the species and candidate species in the *Burkholderia cepacia* species complex (Baldwin *et al.*, 2005) and to resolve *Vibrio* species (Thompson *et al.*, 2005). A major limitation of this approach is that MLST protocols are often designed for single species, frequently those of clinic relevance (http://www.mlst.net/). MLST protocols and the respective sequence databases for environmental isolates are not so common.

In the case of *Acinetobacter* spp., although a MLST protocol only exists for *A. baumannii* (http://pubmlst.org/abaumannii/), the sporadic use of phylogenetic analysis based on protein-encoding genes such as *rec*A, *gro*EL, *hsp*7.5, *rpo*B, *rpo*D and *gyr*B for the classification at the intrageneric level, is reported in literature (Tab. 2) (Bartual, *et al.*, 2005, Haake *et al.*, 1997, La Scola *et al.*, 2006, Pai *et al.*, 1997, Yamamoto & Harayama, 1996, Yamamoto & Harayama, 1998). In this genus, MLST approach, even when based on a reduced number of gene sequences, has proved to offer a higher degree of resolution than that based on the 16S rRNA gene sequence analysis. This is due to the faster evolution of protein-encoding genes than the 16s rRNA gene (Ochman & Wilson, 1987, Yamamoto & Harayama, 1998). However, the unavailability of the sequences of some genes for recently described *Acinetobacter* species exclude the use of those gene sequences for comparing different species of *Acinetobacter* (Nemec *et al.*, 2001, Nemec *et al.*, 2003). As noted by different authors, in the genus *Acinetobacter*, the analysis of nucleotide sequences of essential protein-encoding genes is effective for genomic species identification and extremely useful for the determination of branching orders in evolution of this group (Krawczyk, *et al.*, 2002, Nemec, *et al.*, 2009, Yamamoto *et al.*, 1999).

OBJECTIVE AND WORK STRATEGY

The main objective of this study was to examine the persistence and possible routes of dispersion of *Acinetobacter* spp. in the urban water cycle. A collection of 254 isolates of *Acinetobacter* spp. recovered from different types of water (underground water, surface water and drinking water), were analyzed in two phases. In the initial screening, isolates were presumptively identified using an *Acinetobacter* spp. specific 16S rRNA primer set. After this screening, a mini-MLST approach was used in order to achieve the identification to the species level and, whenever possible, patterns of intra-specific variation. The antibiotic resistance profiles of the isolates were also determined. The mini-MLST patterns and the antibiotic resistance profile were analyzed as a function of the isolation site, aiming the tracking of the isolates in the environment and the inference about the persistence and possible paths of dispersion of *Acinetobacter* spp. through the urban water cycle.

MATERIAL AND METHODS

Water sampling

The bacterial strains examined in this study were isolated from a water treatment plant (WTP), responsible for the water treatment and supply of drinking water to a population of about 1.5 million of inhabitants, and from 11 household taps (from 11 houses) with a sporadic use (1-4 times a month) and one from a health care facility. The isolates included in this study were recovered in two sampling campaigns from tap water and in one from the WTP. The WTP supplies different water distribution systems (WDS), four of which represented in the current study. In Figure 2 is schematized the distribution of WTP, the WDS and the taps. WDS 1 supplies taps no. 2, 8, 9 and the hospital tap; the WDS 2 supplies taps no. 1, 5 and 11; the WDS 3 supplies taps no. 3, 4, 6 and 10; and the WDS 4 supplies tap 7.

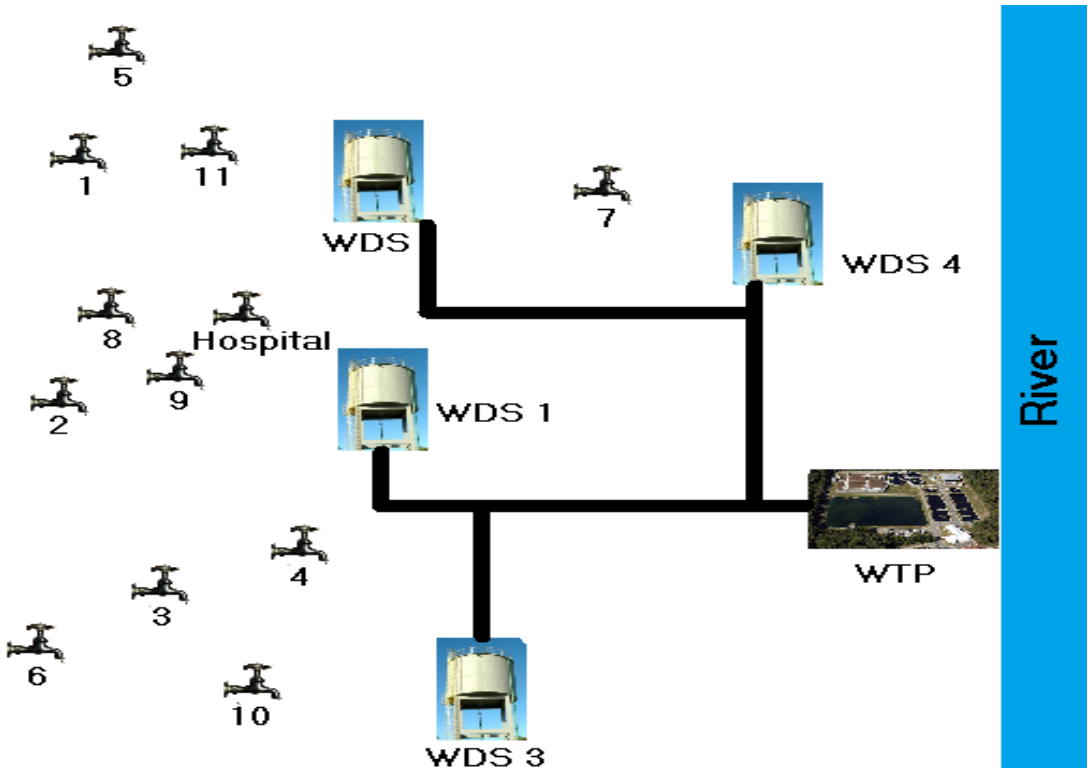

Figure 2. Map of distribution of the taps and the WTP under study.

The names attributed to the isolates were composed by 2 letters followed by a number. The letters stand for the site and culture medium of isolation, respectively. In the case of the tap's isolates the letter following the number indicates the sampling time (A- first sampling; B- second

sampling) (Table 2). In sites A, B and C the sampling was performed in 20/11/2007 and in site E in 15/01/2008. The first sampling of taps water occurred during April of 2009, while the second occurred during September of 2009.

Table 2. Designations of the isolates names

			Designation
Sampling site	Captation	Surface water	A
		Underground water	B
	Disinfection	After ozonation	C
		Before the second chlorination tank	E
	Taps		T
	Hospital Tap		M
Culture medium	mFC		F
	TTC		T
	PIA		P
	R2A		R
	BEA		E

The culture media used were mFC (Difco, composition per liter: 6 g pancreatic digest of casein, 9 g protease peptone no. 3.3 g yeast extract, 12.5 g lactose, 1.5 g bile salts no. 3.5 g sodium chloride, 15 g agar, 0.1 g aniline blue), TTC (Oxoid, composition per liter: 10 g peptone; 6 g yeast extract; 5 g meat extract; 20 g lactose; 0.05 g bromothymol blue; 0.1 g Tergitol-7; 13 g agar supplied as 2 ml of filtered 0.125% aqueous solution of tri-phenyltetrazolium chloride SR0148A from Oxoid), PIA (Difco, composition per liter: 20 g peptone; 1.4 g magnesium chloride; 10 g potassium sulphate; 25 mg Irgasan™; 13.6 g agar), R2A (Difco, composition per liter: 0.5 g yeast extract; 0.5 g proteose peptone no. 3; 0.5 g casamino acids; 0.5 g dextrose; 0.5 g soluble starch; 0.3 g sodium pyruvate; 0.3 g dipotassium phosphate; 0.05 g magnesium sulfate; 15 g agar) and BEA (Pronadisa, composition per liter: 3 g beef extract; 5 g peptone; 40 g ox bile; 1 g esculin; 0.5 g ferric citrate, 15 g agar).

Preliminary characterization of the bacterial isolates

Bacterial isolates were cryopreservated at -80°C in Luria-Bertani broth (composition per liter: 10 g tryptone; 5 g yeast extract; 10 g NaCl) with 15% (p/v) of glycerol. For the first screening,

aiming the selection of *Acinetobacter* spp., bacteria were cultivated on PCA (Liofilchem, composition per liter: 5 g of tryptone; 1 g of glucose; 2.5 g of yeast extract; 15 g of agar) and tested for Gram staining, cytochrome C oxidase and catalase presence. After these assays, only isolates that were Gram-negative coccobacilli, oxidase-negative and catalase-positive were selected for further analysis. These isolates were screened using an *Acinetobacter* spp. – specific PCR reaction.

PCR-based screening of presumable *Acinetobacter* spp.

The *Acinetobacter* spp. isolates were identified by PCR using an *Acinetobacter* spp. specific 16S rRNA primer set Ac436F (5'TTTAAGCGAGGAGGAGG) and Ac676R (5'ATTCTACCATCCTCTCCC) (Vanbroekhoven *et al.*, 2004). The annealing temperatures and GC content are 52.8°C and 53.7°C with 52.9% and 50%, respectively, creating a fragment of 240bp. Crude cell lysates (low density suspension in 100 mL of ultra pure water (2) vortex for 15 seconds (3) 10 minutes at 95°C (4) 5 minutes on ice (5) centrifuge for 2.5 minutes at 13000 rpm (6) save the supernatant at – 20°C) were used as DNA template in a PCR reaction, as follows: 1.25 µL of 10X buffer with KCl (Fermentas), 0.75 µL of 1.5 mM $MgCl_2$ (Fermentas), 1.25 µL of dNTP's (1 mM), 0.25 µL of each primer (10 µM), 0.35 µL of Taq polymerase 1U/µL (Fermentas), 0.5 µL of template DNA and 7.9 µL of ultra pure water in order to obtain a 12.5 µL reaction.

PCRs were performed in a thermocycler Tprofessional Basic Gradient (Biometra) with the following programme: (1) initial denaturation at 95°C for 5 min; (2) 30 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 30 sec and polymerization at 72°C for 40 sec; (3) extension at 72°C for 4 min. For size comparison and confirmation of the reaction success, a positive control (DNA template from *A. baumannii* CCUG 19096^T), as well as two negative controls (DNA template from *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* DSM 1117) were included in each run.

To confirm the reliability of the method, 10% of the isolates yielding a negative or positive response for the PCR reaction were identified by the 16S rRNA gene sequence analysis as described by Ferreira da Silva *et al.* (2007). The 16S rRNA gene sequences were compared with

others available in public databases using the software FASTA (http://www.ebi.ac.uk/Tools/fasta33/).

Antibiotic resistance phenotypes

Antibiotic resistance phenotypes were tested based on the Kirby–Bauer method according to standard recommendations (Clinical and Laboratory Standards Institue, 2007). Bacterial suspensions with an OD of 0.2, at 610 nm, were spread unanimously on Mueller Hinton agar plate (Oxoid, composition per liter: 300 g dehydrated infusion from Beef; 17.5 g of Casein hydrolysate; 1.5 g of Starch and 17 g of agar) using a sterile cotton swab. Antibiotic discs of amoxicillin (AML, 25 μg), gentamicin (GEN, 10 μg), ciprofloxacin (CIP, 5 μg), sulphamethoxazole/trimethoprim (SXT, 23.75/1.25 μg), tetracycline (TET, 30 μg), cephalothin (CP, 30 μg), meropenem (MEM, 10 μg), ceftazidime (CAZ, 30 μg), ticarcillin (TIC, 75 μg), colistin sulphate (CT, 50 μg), sulphamethoxazole (SUL, 25 μg) and streptomycin (STR, 10 μg) (all Oxoid) were placed on the surface of each inoculated plate. After 24 h of incubation at 37°C, except some isolates that were not able to grow at 37°C and instead were incubated at 30°C, the diameters of antibiotic inhibition of growth were measured and recorded as susceptible (S), intermediary (I) or resistant (R). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* DSM 1117 were used as controls in each experimental set.The adopted interpretation criteria based on inhibition zone diameters were as follows (mm): AML 25 μg: susceptible (S) ≥21, resistant (R) <14; TIC 75 μg: S≥20, R≤14; CP 30 μg: S>14, R≤14; CAZ 30 μg: S≥18, R≤14; MEM 10 μg: S≥16, R≤13; CT 50 μg: S>10, R≤10; SUL 25 μg: S≥16, R<12; SXT 25 μg: S≥16, R<10; CIP 5 μg: S≥21, R<15; TET 30 μg: S≥15, R<11; GEN 10 μg: S≥15, R≤12; STR 10 μg: S≥15, R<13. The inhibition zones observed for these organisms were compared with expected values. The average deviation of the diameters of inhibition zones measured for the control strains ranged between 0.1 and 0.2 mm.

Analysis of partial sequences of housekeeping genes

In order to assess the intra-specific genetic variability of the isolates under study, the partial sequences of two housekeeping genes were analyzed – *rpo*B and *rec*A.

Amplification of *rpo*B gene

The partial sequence of the gene *rpo*B was amplified with the primers Ac696F (5' TAYCGYAAAGAYTTGAAAGAAG) and Ac 1598R (5' CGBGCRTGCATYTTGTCRT) (La Scola*, et al.*, 2006). The annealing temperatures and GC content are 60°C and 58°C with 34% and 53%, respectively, creating a fragment of 827bp corresponding to the region 2946-3769 according to the rpoB sequence o *A. baumanni*. The PCR was performed with 5 µL of 10X buffer with KCl (Fermentas), 3.6 µL of 1.5 mM $MgCl_2$ (Fermentas), 10 µL of dNTP's (1 µM), 1 µL of each primer (10 µM), 0.625 µL of Taq polymerase 1 U/µL (Fermentas), 2 µL of template DNA and 28.15 µL of ultra pure water in order to obtain a 50 µL reaction. For size comparison and confirmation of the reaction success, a positive control (DNA template from *A. baumannii* CCUG), as well as a negative control (ultrapure water was used instead of DNA template), was included in each run. PCRs were performed in the with the following programme: (1) initial denaturation at 95°C for 2 min; (2) 35 cycles of denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec and polymerization at 72°C for 1 min; (3) extension at 72°C for 10 min.

Amplification of *rec*A gene by PCR

The partial sequence of the gene *rec*A was amplified with the primers RA1 (5'CCTGAATCTTCTGGTAAAAC) and RA2 (5'GTTTCTGGGCTGCCAA ACATTAC) (Nowak & Kur, 1996). The annealing temperature and GC content of RA1 and RA2 are 54.2°C with 42.4% and 60.6°C with 47.8%, respectively, creating a fragment of 362 bp corresponding to the region 238-599 of *A. baumannii* gene. The PCR was performed with 12.5 µL of Taq PCR MasterMix Kit 250 Units (Quiagen), 2.5 µL of each primer (10 µM), 2.5 µL of ultra pure water and 5 µL of template DNA in order to obtain a 25 µL reaction. All samples were run in duplicates (2 x 25 µL reactions) and in one of the reactions the DNA was diluted with ultrapure water to 1/10. For size comparison and confirmation of the reaction success, a positive control (DNA template from *A. baumannii* CCUG 19096^T), as well as a negative control (ultrapure water was used instead the DNA template), was included in each run. PCRs were performed in the thermocycler (MJ Research PTC-200) with the following programme: (1) initial denaturation at 95°C for 2 min; (2) 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 1min and polymerization at 72°C for 2 min; (3) extension at 72°C for 10 min.

Electrophoresis and PCR product purification

In order to confirm the presence of desired PCR product, DNA fragments were resolved by electrophoresis, in a horizontal slab gel apparatus (BioRad mini-sub cell GT). The gel was made by melting Ultrapure™ Agarose (Invitrogen) in 1X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and the running buffer used was TBE at the same concentration. To a volume of 5 µL of PCR product was added 2 µL of loading buffer (Fermentas). Electrophoresis was carried for 30 minutes at 80V. The gels were stained in a solution of 10 $mg.mL^{-1}$ EtBr for 30 minutes and were observed in the GeneFlash transilluminator (Syngene) under a UV light.

In order to remove the free nucleotides, primers and other impurities of the amplified samples, the Qiagen DNA Purification Kit (Qiagen) was used according to the supplier's instructions, and the purified PCR products were stored at -20°C.

DNA sequencing analysis

The reactions for sequencing analysis were performed, using the PCR-amplification primer pair using 2 µL of Big dye terminator (Applied Biosystems), 2 µL of 2.5X Big dye sequencing buffer (Applied Biosystems), 1 µL of primer, 13 µL of ultrapure water and 2 µL of DNA template in order to obtain a 20 µL reaction. PCR steps were as follows: (1) initial denaturation for 5 min; (2) 25 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 15 sec and at 60°C for 2.5 min; (3) hold at 4°C until ready to purify.

To purify the PCR product, 2 µL of 3 M sodium acetate and 50 µL of 95 % ethanol was added to the PCR product, and left at room temperature for 20 min. After being centrifuged for 20 min at 13000 rpm, the supernatant was removed and 250 µL of 70% ethanol was added and centrifuged again for 5 min at 13000 rpm. Then all supernatant was removed carefully and the DNA extracts were heated for 2 min at 95°C to be sure that all ethanol was removed.

The DNA extracts were dissolved in 15 µL of formamid (Applied Biosystems) and transferred to a 96 well plate, the sequencing was carried by a fluorescence-based capillary electrophoresis system, (Applied Biosystems 3130XL Genetic Analyzer).

The partial *rpo*B and *rec*A gene sequences were checked and edited manually using the software Bionumerics (v6.1 Applied Maths). Also the sequence alignments and the phylogenetic trees were made with Bionumerics using neighbour-joining method based on the model of Jukes & Cantor and Maximum Parsimony.

RESULTS

Preliminary characterization and *Acinetobacter* spp. screening

On basis of preliminary phenotypic characterization, a total of 254 Gram-negative, catalase-positive, oxidase-negative, coccobacilli were considered presumable *Acinetobacter* and were selected for further analysis. These isolates were screened with an *Acinetobacter*-specific primers set, which target a signature region of the 16S rRNA gene, with 240 bp (Figure 3).

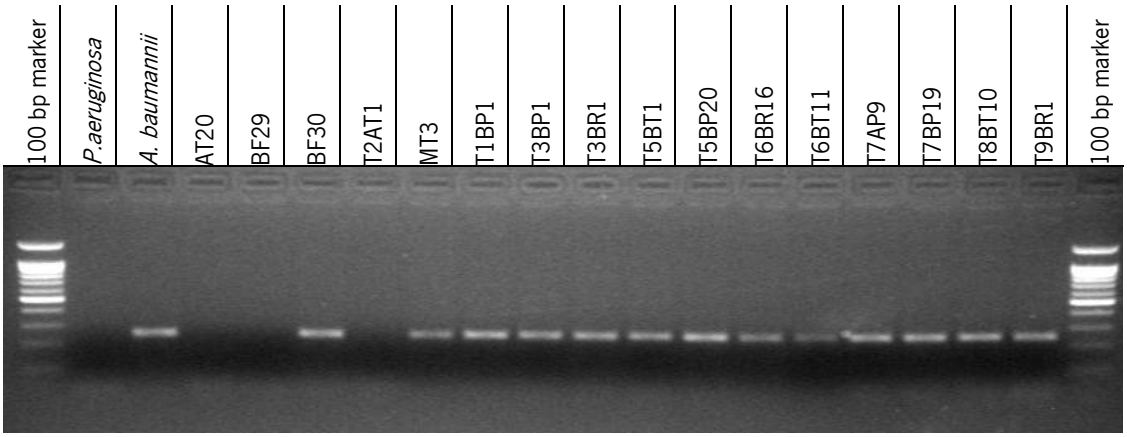

Figure 3. Agarose gel with *Acinetobacter* spp. specific 16S rRNA PCR products from the isolates.

This procedure led to the inclusion of 179 isolates, out ot the 254, in the *Acinetobacter* genus. All the isolates from taps 2 and 4 were excluded after this screening (Figure 4).

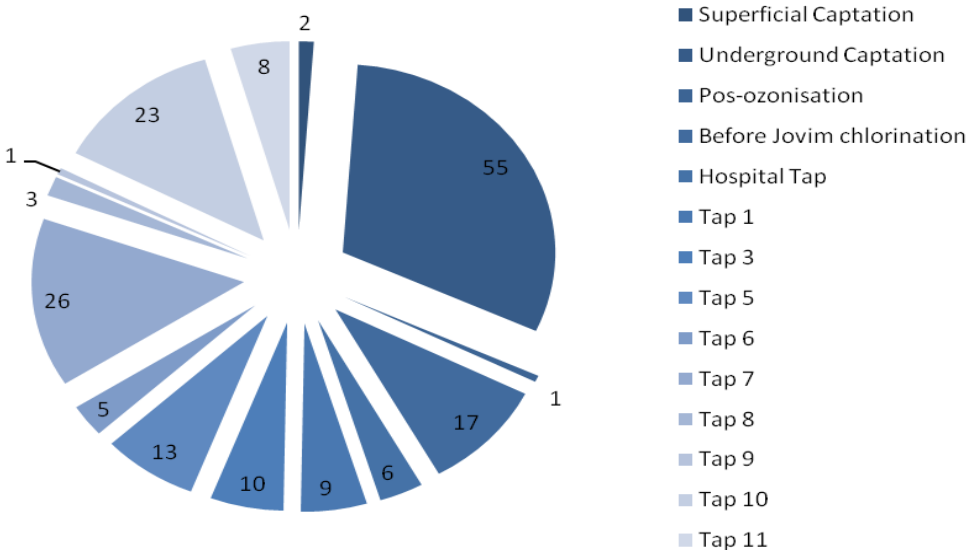

Figure 4. Distribution of the 179 isolates studied by the different sampling sites.

Antibiotic resistance phenotype

The characterization of the antibiotic resistance phenotypes showed that, among the 179 isolates, no resistance to gentamicin, ciprofloxacin, meropenem, colistin sulphate and ticarcillin was observed. Resistance to cephalotin was the most common phenotype, presumably intrinsic in some groups. A total of 41 isolates (22%) was susceptible to all the antibiotics tested. According to their antibiotic resistance phenotypes, isolates could be grouped in eight categories (Table 3).

Table 3. Description of the different phenotypes and prevalence among the isolates

Antibiotic resistance pattern	No of isolates	Antibiotics						
		AML	CP	CAZ	SUL	SXT	TET	STR
1	41	S	S	S	S	S	S	S
2	96	S	R	S	S	S	S	S
3	1	S	S	R	S	S	S	S
4	2	S	S	S	R	S	S	S
5	21	S	R	S	S	S	R	S
6	2	R	R	S	S	S	S	S
7	15	R	R	S	S	S	S	R
8	1	R	R	S	R	R	S	S

All intermediates phenotypes were considered as susceptible
To the antibiotics GEN, CIP, MEM, CP and TIC no resistance phenotypes were registered

-Analysis of the partial nucleotide sequences of the genes *rpo*B and *rec*A

The partial sequences of the genes *rpo*B and *rec*A were compared and with each set of nucleotide sequences were produced two dendrograms which facilitate the assessment of the relationship between the isolates under study and the respective affiliation to the different *Acinetobacter* species. With this objective, the type strains of the 22 validly named *Acinetobacter* species were also included in the dendrograms (Figure 5 Figure 6). This procedure revealed that the isolates were related with the species *A. lwoffii* (n=56), *A.johnsonii* (n=52), *A. junii* (n=24), *A. parvus* (n=24); *A. baumannii* (n=15), *A. beijerinckii* (n=3), *A. tandoii* (n=2), *A. bouvetii* (n=1), *A. baylyi* (n=1) and *A. calcoaceticus* (n=1). After assigning, on basis of the *rop*B gene sequence analysis, the isolates to the different *Acinetobacter* species, a deeper analysis was made in order to get further insights into their diversity and possible clonality. With this objective, sequence matches were made on basis of pairwise comparisons and the isolates with identical

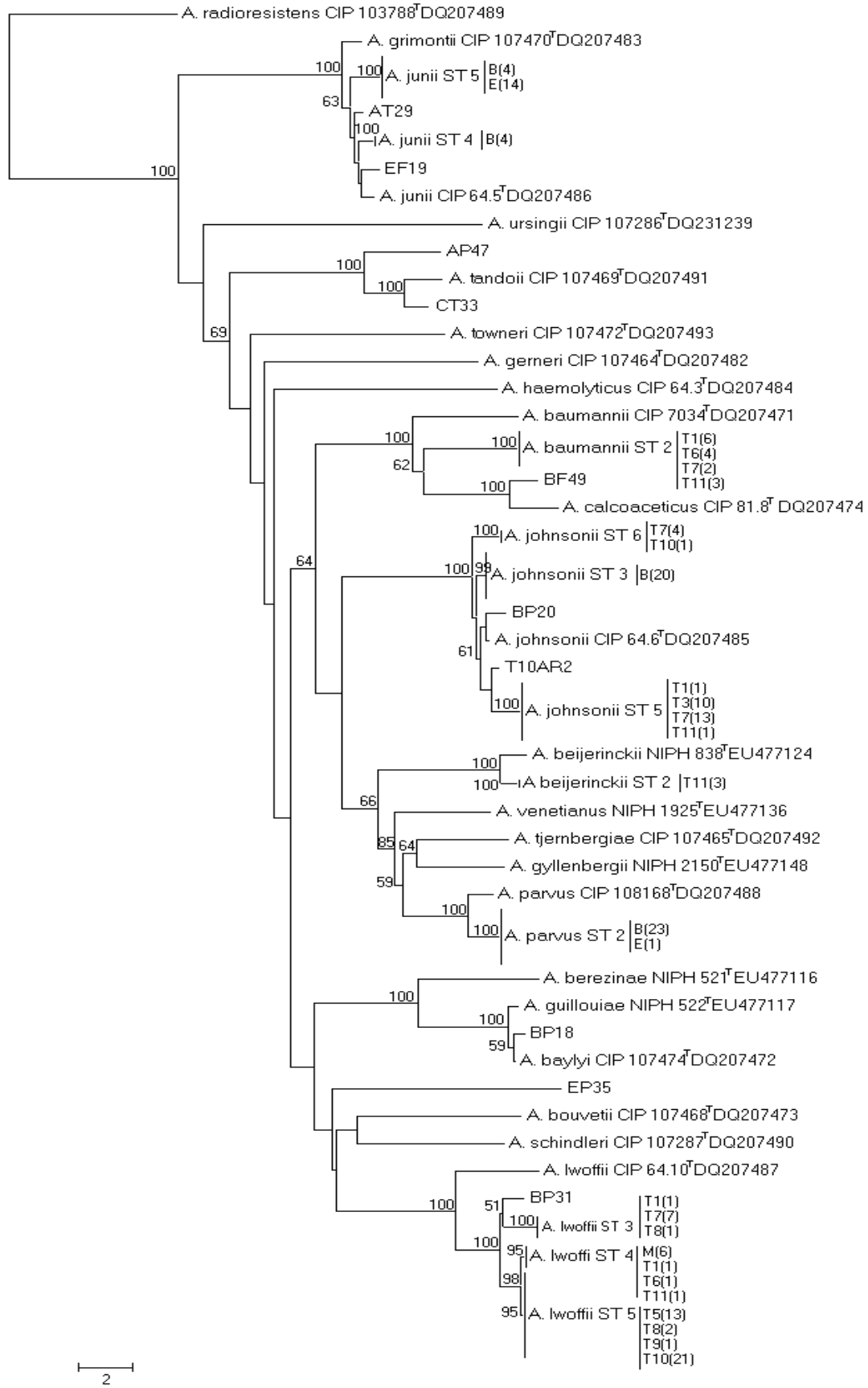

Figure 5. Dendrogram expressing the *rpo*B similarity between isolates was generated using the neighbour-joining method based on the model of Jukes & Cantor with 1000 Bootstrap simulations. Scale bar represents 2 nucleotide substitutions per 100 positions

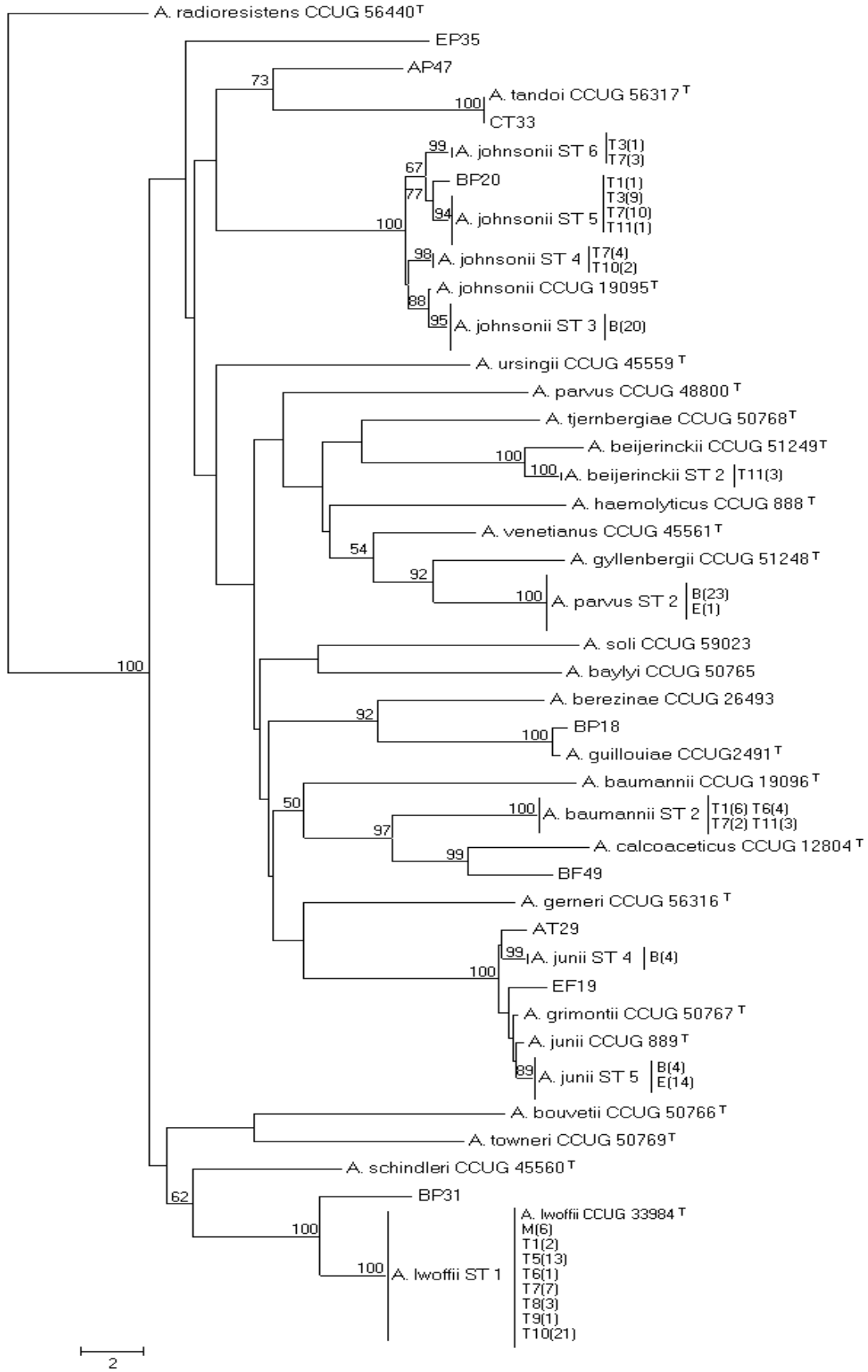

Figure 6. Dendrogram expressing the *rec*A similarity between isolates was generated using the neighbour-joining method based on the model of Jukes & Cantor with 1000 Bootstrap simulations. Scale bar represents 2 nucleotide substitutions per 100 positions.

nucleotide sequences were classified in the same sequence type (ST) group. The names of the ST groups include a reference to the species to which the isolates belong and the group ST 1 comprises always the type strain of the respective species.

Table 4. *rpo*B and *rec*A sequence types crossed with local o isolation and antibiogram phenotypes

Site	Group	Sequence Type Group (n)	*rpo*B Sequence Type (n)	*rec*A Sequence Type (n)	Antibiogram Phenotype (n)
A	*A. junii*	ju2(1)	2(1)	2(1)	2 (1)
	A. tandoii	ta3(1)	3(1)	2(1)	2(1)
B	*A. lwoffii*	lw2(1)	2(1)	2(1)	1(1)
	A. junii	ju4(4) ju5(4)	4(4) 5(4)	4(4) 5(4)	1(1) 2(7)
	A. johnsonii	jo2(1) jo3(20)	2(1) 3(20)	2(1) 3(20)	1(1) 2(20)
	A. parvus	pa2(23)	2(23)	2(23)	2(2) 5(21)
	A. baylyi	ba2(1)	2(1)	2(1)	6(1)
	A. calcoaceticus	ca2(1)	2(1)	2(1)	6(1)
C	*A. tandoii*	ta2(1)	2(1)	1(1)	1(1)
E	*A. junii*	ju3(1) ju5(15)	3(1) 5(15)	3(1) 5(15)	2(16)
	A. parvus	pa2(1)	2(1)	2(1)	2(1)
	A. bouvetii	bo2(1)	2(1)	2(1)	3(1)
M	*A. lwoffii*	lw4(6)	4(6)	1(6)	2(6)
T1	*A. lwoffii*	lw3(1) lw4(1)	3(1) 4(1)	1(2)	2(2)
	A. baumannii	bm2(6)	2(6)	2(6)	7(6)
	A. johnsonii	jo5(1)	5(1)	5(1)	2(1)
T3	*A. johnsonii*	jo5(9) jo6(1)	5(10)	5(9) 6(1)	2(10)
T5	*A. lwoffii*	lw5(13)	5(13)	1(13)	1(13)
T6	*A. lwoffii*	lw4(1)	4(1)	1(1)	1(1)
	A. baumannii	bm2(4)	2(4)	2(4)	7(4)
T7	*A. lwoffii*	lw3(7)	3(7)	1(7)	2(7)
	A. baumannii	bm2(2)	2(2)	2(2)	7(2)
	A. johnsonii	jo5(10) jo6(3) jo7(4)	5(13) 6(4)	5(10) 6(3) 4(4)	2(17)
T8	*A. lwoffii*	lw3(1) lw5(2)	3(1) 5(2)	1(3)	1(2) 4(1)
T9	*A. lwoffii*	lw5(1)	5(1)	1(1)	1(1)
T10	*A. lwoffii*	lw5(21)	5(21)	1(21)	1(20) 4(1)
	A. johnsonii	jo4(1) jo7(1)	4(1) 6(1)	4(2)	2(1) 8(1)
T11	*A. lwoffii*	lw4(1)	4(1)	1(1)	2(1)
	A. baumannii	bm2(3)	2(3)	2(3)	7(3)
	A. johnsonii	jo5(1)	5(1)	5(1)	2(1)
	A. beijerinckii	be2(3)	2(3)	2(3)	2(3)

The ST formed on basis of *rpo*B and *rec*A nucleotide sequences coincided in the cases of the groups A. junii, A. baumannii, A. beijerinckii and A. parvus. In the group A. johnsonii, although *rec*A and *rpo*B led to the definition of 5 ST, it comprised a different set of isolates in each case. In the group A. lwoffii, *rec*A, showed a lower resolution forming only 2 ST, in contrast with the 4 formed with the *rpo*B sequence. A similar finding was observed in the group A. tandoii, in which rpoB formed 2 ST while recA did not allow the differentiation of ST.

The combination of the *rpo*B- and *rec*A-based ST did not bring additional differentiation, except in the case of the A. johnsonii group ( ). The distribution of the same ST by different sampling sites, showed that ST lw3, lw4, lw5, ju5, bm2, jo5, jo6, jo7 and pa2 were detected in different taps or different sampling site of the WTP (Figure 7). The same ST was never found in the WTP and taps. The same antibiogram pattern was distributed by different sampling sites and corresponded to different ST ( ).

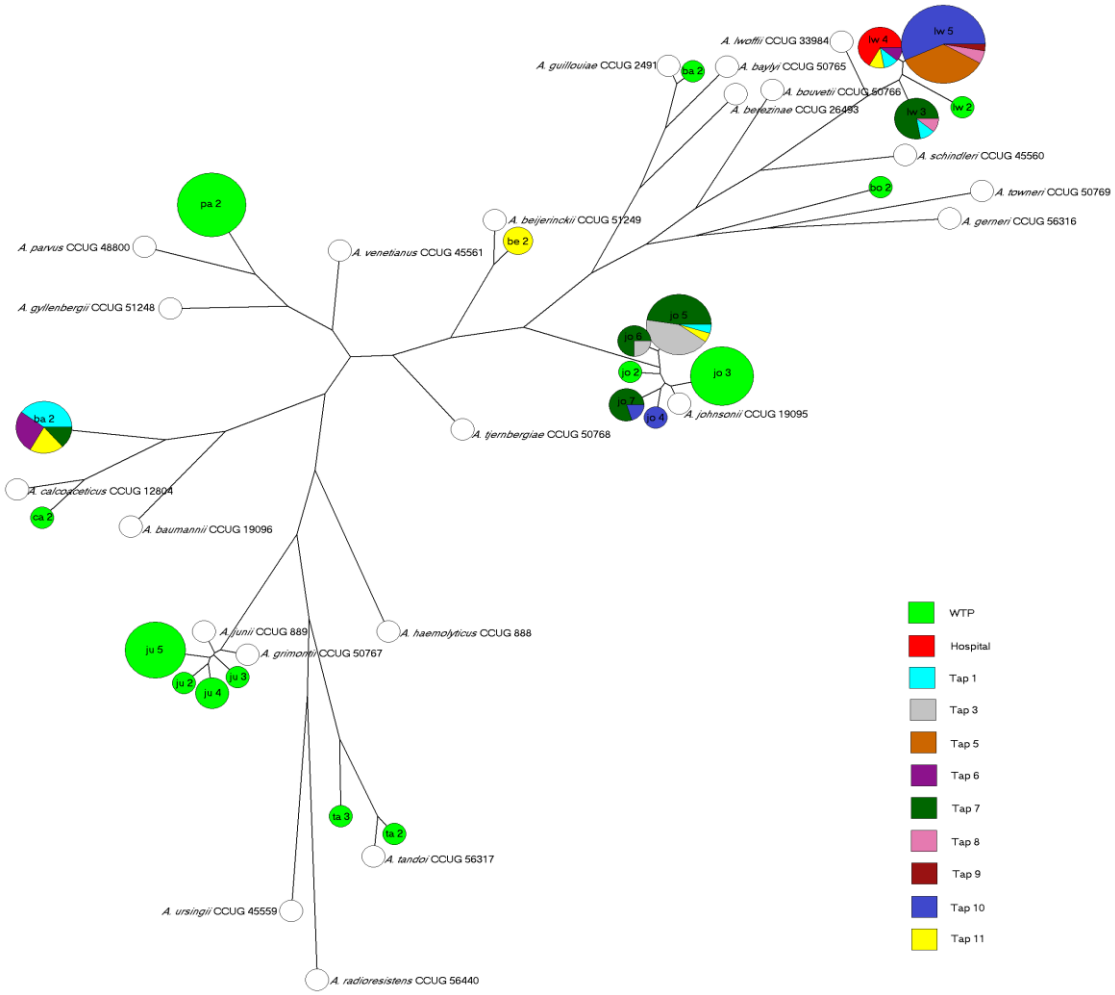

Figure 7. Maximum Parsimony tree constructed on basis of the *rpo*B and *rec*A concatenated sequences, with the isolates classified according to the isolation site (colors) and ST (circles).

DISCUSSION AND CONCLUSIONS

Acinetobacter spp. are known for being widespread in the nature, largely due to their capability to grow at the expenses of a great number of carbon sources (Baumann*, et al.*, 1968, Bifulco*, et al.*, 1989). These properties put *Acinetobacter* spp. in the urban water cycle scenario, with the presence of members of this genus being described in treated water and in groundwater systems (Bifulco*, et al.*, 1989, Lacroix & Cabelli, 1982). These previous evidences support the current results that report the isolation of *Acinetobacter* spp. from the water distribution system and from tap water, even in two different sampling campaigns. The sampling from taps with reduced use aimed at targeting bacteria which may be involved in biofilm structures, as the water flow may influence the biofilm growth. There are also other aspects like the materials of WDS are comprised, dissolved organic carbon, amount of disinfectant, temperature, which can define the bacterial abundance and diversity (Bai *et al.*, 2010, Manuel *et al.*, 2007).

This work aimed to study the dispersion and the persistence of *Acinetobacter* spp. in the urban cycle of water. First will be discussed the dispersion.

After determining the *Acinetobacter* spp. isolates through the analysis of 16S rRNA gene the isolates number was 179, being 42% of those isolates from the WTP and the other 58% to the 12 taps, which were supplied by that WTP. Given the limitations of the 16S rRNA gene sequence analysis to determine species affiliation in the genus *Acinetobacter*, it was opted to make this identification to the species level on basis of the analysis of the partial sequence of the gene *rpo*B. This gene sequence has been widely used for species differentiation in this genus, often substituting the universal phylogenetic marker 16S rRNA gene (Gundi *et al.*, 2009, La Scola*, et al.*, 2006, Nemec*, et al.*, 2010). On basis of this analysis, the isolates were distributed by the species *A. lwoffii*, *A.johnsonii*, *A. junii*, *A. parvus*; *A. baumannii*, *A. beijerinckii*, *A. tandoii*, *A. bouvetii*, *A. baylyi* and *A. calcoaceticus*. Besides the validly named species, several genomic species have been proposed within the genus *Acinetobacter*. Even though these genomic species have no taxonomic value, they correspond to coherent phylogenetic groups. Part of the isolates included in the current study was, in fact, more closely related with such genomic species than with the validly named groups. This was evident with the isolates included in the group of *A. baumannii,* which yielded the highest similarity score with *Acinetobacer*

genomic species 3, presenting lower *rpo*B gene sequence similarity values with *A. baumannii* CIP 7034^{T} (92.5 %). Another example was given by a single isolate (EP35), which evidenced low *rpo*B and *rec*A sequence similarity values (<90 %) with other sequences available in public databases.

Looking the distribution of species in the cycle of water under study, it is found that *A. tandoii*, *A. calcoaceticus*, *A. parvus*, *A. junii*, *A. bouvetii* and *A. baylyi* were only sampled in the WTP while *A. baumannii* and *A. beijerinckii* were only sampled in taps. The *A. johnsonii* and *A. lwoffii* were the only species with representatives sampled from both sites but with different STs.

Comparing the abundance of *Acinetobacter* spp. in the different sampling points, it was observed that from the underground captation (B) was recovered the largest number (55). Water ozonation led to the reduction of the abundance of *Acinetobacter* spp., with a single isolate being recovered after this disinfection stage. The re-growth and/or the re-introduction of *Acinetobacter* spp. in the water circuit was observed in a downstream sampling site, before the second chlorination tank (E), from which were recovered 17 isolates. In the following stages of water treatment until the water leave the plant it was not found any *Acinetobacter* spp. isolate. The analysis of the *rpo*B- and *rec*A-based dendrograms (Figure 5Figure 6) shows that ST ju5 comprises isolates from sites B and E suggesting that *Acinetobacter* spp. may persist after ozonation and disinfection, as Kormas et al. (2010) observed in γ-Proteobacteria.

Looking the distribution of species in the cycle of water under study, it is found that *A. tandoii*, *A. calcoaceticus*, *A. parvus*, *A. junii*, *A. bouvetii* and *A. baylyi* were only sampled in the WTP while *A. baumannii* and *A. beijerinckii* were only sampled in taps. The *A. johnsonii* and *A. lwoffii* were the only species with representatives sampled from both sites. *rpo*B- and *rec*A-based dendrograms analysis (Figure 5Figure 6) shows that *Acinetobacter* spp. reaching the taps did not have origin in the WTP, as no identical ST was observed in both types of sampling sites. Nevertheless, a common source of tap water *Acinetobacter* spp. is hinted by the observation of the same ST in different taps and WDS. It is the case of ST jo5 which comprises isolates from 3 different WDS. Although these findings suggest that the WTP is not the source of *Acinetobacter* spp. in the taps, is important to note that the WTP was sampled only once and one year and half earlier than the taps.

In an attempt to assess the persistence of *Acinetobacter* spp. in the water samples, a comparison between two distinct sampling periods was made. While from the first sampling campaign, which took place in April of 2009, only seven isolates were obtained, three from tap 10 and four from tap 7, in the second sampling period, in September of 2009, a total of 91 *Acinetobacter* spp. isolates was recovered. These results suggest that the abundance and/or culturability of *Acinetobacter* spp. may increase considerably during the summer period. In respect to persistence, except the isolate T10AT20 which was included in the ST lw5, together with isolates from the second sampling campaign, no identical *rpo*B or *rec*A nucleotide sequences were observed in different sampling periods. The inclusion of strain T10AT20 in that ST may result from the poor resolution of the method used, as it is possible to distinguish this isolate from the others through the antibiogram phenotype (7). Taking these evidences it is plausible to admit that the presence of *Acinetobacter* spp. in the urban water cycle rise during the summer period and vanishes during winter, with a new colonization in the following year.

The MLST scheme provides a high level of resolution and an excellent tool for studying the population structure (Bartual*, et al.*, 2005), and in this work, trying to reduce the spent time and costs was designed a mini-MLST scheme for *Acinetobacter* spp. With this purpose, two housekeeping genes, *rpo*B and *rec*A, described as having a good resolution at species level for *Acinetobacter* genus (Krawczyk*, et al.*, 2002, La Scola*, et al.*, 2006), were analyzed. When compared to *rec*A, the gene *rpo*B proved to have higher resolution to discriminate isolates within the species in *A. lwoffi* and *A. tandoii*, originating more STs. In the species *A. lwoffii* the *rpo*B gene led to the definition of five STs, while *rec*A allowed the differentiation of only two groups. In *A. johnsonii* group both genes created 6 STs, but grouping differently the isolates, being at the end 7 the number of STs in the group.

High proportions of *Acinetobacter* strains have become resistant to older antibiotics (Misbah *et al.*, 2004, Towner, 2009, Turton *et al.*, 2010). Indeed, the resistance to clinically doses of antibiotics is not uncommon among members of the genus *Acinetobacter*. A good example is given by the antibacterial drug cephalothin, for which acquired resistance has been reported (Bergogne-Berezin & Towner, 1996), and which yielded high rates of resistance in the current study. In fact, about 75% of the isolates demonstrated resistance to this cephalosporin. Resistance to amoxicillin and cephalothin was demonstrated in WTP as in taps isolates equally

distributed, while only in isolates from the water taps was demonstrated resistance to sulphamethoxazole/trimethoprim, sulphamethoxazole and streptomycin. Resistance to tetracycline and ceftazidime was observed exclusively in the WTP. Although a higher diversity of resistance phenotypes was observed among the tap water isolates, the susceptibility to all drugs was also more common in these organisms. *A. lwoffii* was the group that yielded lower resistance rates, with 67% of its isolates being susceptible to all tested antibiotics. *A. beiherinckii*, *A. junii* and *A.tandoii* only demonstrate resistance to cephalothin. Resistance to this cephalosporin and tetracycline was common among the *A. parvus* isolates. The only isolates demonstrating resistance to streptomycin were related to the species *A. baumannii*, often together with amoxicillin and cephalothin resistance. Resistance to ceftazidime was not common among the environmental isolates and was demonstrated in isolate EP35, which species affiliation is not certain. Thus further analyses are necessary to explain this resistance phenotype. Nevertheless, in general, most of the isolates were susceptible to at least 11 of the 12 antibiotics tested. This low rates of antibiotic resistance are probably due to the fact that the isolates examined in this study were from environmental samples and presumably not subjected to selective pressure, as those imposed by antibiotics. In general, the antibiotic resistance patterns were in concordance, but with a lower resolution, with STs defined on basis of the mini-MLST. However, seven isolates, namely the isolate T10AT20, presented differentiated antibiotic resistance patterns, hinting processes of resistance acquisition and/or emergence.

The use of additional gene sequences for strain differentiation will increase the power of resolution of the mini-MLST scheme. In this respect, gene gyrB seems to be a good candidate to the place having good results of resolution in *Acinetobacter* spp. (Yamamoto*, et al.*, 1999).

FUTURE WORK

The inclusion of isolates recovered in more sampling campaigns and, thus, the enlargement of number of isolates under investigation, will bring further insights into the trends observed in the current study in what respects dispersion and persistence. The analysis of additional sampling sites, namely the water reservoirs that supply the taps could also be of great relevance in this study as it would contribute to assess the origin of the *Acinetobacter* spp. detected in the tap water. One of the major drawbacks in the current study was the poor resolution that the use of only two housekeeping gene sequences may have. The inclusion of at least an additional gene sequence, namely of *gyr*B, which is known to resolve the different *Acinetobacter* species, will contribute to improve the resolution power of the mini-MLST scheme. The improvement of the mini-MLST resolution will bring new clues about the *Acinetobacter* spp. intra-specific variation and also about the paths of antibiotic resistance acquisition and/or selection throughout the urban water cycle.

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