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ORIGINAL PAPER

Molecular characterization of *Acanthamoeba* isolated in water treatment plants and comparison with clinical isolates

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Abstract A total of 116 samples (44 clinical specimens and 72 environmental samples) have been analyzed for the presence of *Acanthamoeba*. The environmental samples (ESs) were collected from four drinking water treatment plants (DWTP, $n=32$), seven wastewater treatment plants ($n=28$), and six locations of influence ($n=12$) on four river basins from the central area of Spain (winter–spring 2008). Water samples were concentrated by using the IDEXX Filta-Max® system. *Acanthamoeba* was identified in 65 of the 72 ESs by culture isolation (90.3%) and 63 by real-time PCR (87.5%), resulting in all sampling points (100%) positive for *Acanthamoeba* when considering both techniques and all the time period analyzed. Nine of the 44 clinical specimens were positive for *Acanthamoeba.* Seventeen *Acanthamoeba* strains (eight from four DWTP and nine from clinical samples) were also established in axenic-PYG medium. Twenty-four of the ESs and the 17 *Acanthamoeba* sp. strains were genotyped as T4/1, T4/8, and T4/9. The eight strains isolated from the DWTP samples were inoculated in nude mouse to ascertain their potential pathogenicity in this model. Animals that were inoculated died or showed central nervous system symptoms 9 days post-inoculation. Examination of immunofluorescence-stained brain and lung tissue sections showed multiple organisms invading both tissues, and re-isolation of throphozoites was successful in these tissues of all infected animals. For the first time, potentially pathogenic *Acanthamoeba* T4 has been detected in 100% of different types of water samples including tap water and sewage effluents in the central area of Spain suggesting a potential health threat for humans especially for the contact lens wearers.

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Introduction

Free-living amoebae (FLA), belonging to the genera *Acanthamoeba*, *Balamuthia*, and *Naegleria* occur worldwide. *Naegleria fowleri* causes an acute infection of the central nervous system (CNS) called primary amebic meningoencephalitis leading to death in most cases, whereas several strains of *Acanthamoeba* and *Balamuthia mandrillaris* can

cause a chronic infection of the CNS, granulomatous amebic encephalitis (GAE), as well as a disseminated infection affecting the skin and lungs, also leading to death. Of these three agents, *Acanthamoeba* is isolated as the etiologic agent of *Acanthamoeba* keratitis (AK) (Visvesvara et al. 2007), but it is indeed more frequently detected in nasal mucosa of healthy people (De Jonckheere and Michel 1988).

As the number of patients suffering from AK rises worldwide, so has the interest in this infection. *Acanthamoeba* has been isolated worldwide from disparate environments such as fresh and salt water, drinking water, contact lens (CtL) washing solutions, ventilation systems, dialysis apparatus, hydrotherapy areas in hospitals, and dental irrigation systems (Booton et al. 2005; Marciano-Cabral and Cabral 2003; Visvesvara et al. 2007). In addition, outbreaks of CtL-associated AK have recently been reported in the USA (Joslin et al. 2007; Visvesvara et al. 2007; Verani et al. 2009).

However, to our knowledge, there are only five reports describing 17 cases of CtL-associated AK in Spain (Cruz et al. 2004; de Miguel et al. 1999; Lopez et al. 2000; Lorenzo-Morales et al. 2007; Perez-Pomata et al. 2006). In addition, there are only three publications that describe cases of GAE (Gene et al. 2007; Peman et al. 2008; Seijo Martinez et al. 2000). Environmental studies on *Acanthamoeba* epidemiology are also rare in Spain, and most of them were carried out on the Tenerife Island, which showed high concentrations of the amoeba in fresh (59%) and salt water (40%) (Lorenzo-Morales et al. 2005a; Lorenzo-Morales et al. 2005b). The aim of this study was to ascertain the presence of *Acanthamoeba* in water samples collected from the central area of Spain and to characterize and compare these isolates with those obtained from clinical samples.

Materials and methods

Samples

Seventeen sample sites on four river basins from the central area of Spain, where livestock, mainly cattle are being raised for milk and meat, were selected (Fig. 1 and Table 1). These sites included four drinking water treatment plants (DWTP), one on each river basin, seven wastewater treatment plants (WWTP), and six locations of influence (LI) (one on the edge of a river, two reservoirs, and three gauging stations). The LIs were selected because of high livestock activity in the area or due to its location downstream from the WWTP. The sampling was done in duplicate during the winter and spring of 2008. For the DWTP, 100 l of water was collected from each site (raw water—at the point of entry and finished water—at the end of the process). We collected 50 l from the WWTP, each at both points, as above and 50 l for the six LIs. The sampling scheme therefore included the initial collection of 16 water samples from DWTP in the winter and 56 water samples in the spring from the entire area under study (DWTP, WWTP, and LI). In all cases, the water samples were concentrated by using the IDEXX® Filta-Max system as per the manufacturer's instructions and following the Environmental Protection Agency of the United States (US EPA) 1623 Method (US EPA 2005). A total of 5 ml was finally eluted from each concentrated sample and fractioned for different analysis. Samples for molecular analysis were kept at −80°C. Additionally, 44 clinical samples were obtained from patients referred to hospitals and clinical laboratories for AK diagnosis, during 2005–2008 that included corneal scrapes, vitreous humor, contact lenses, and contact lens solutions (Table 1).

Acanthamoeba culture

Approximately 80 μl of the 72 concentrated water samples and the 44 clinical samples (corneal scrapes, vitreous humor, contact lenses, and contact lenses solutions) were inoculated onto 2% Neff's saline non-nutrient agar plates seeded with heat-killed *Escherichia coli* and incubated at 28°C. Initial cultures were monitored daily and subcultured by transferring small pieces of agar containing *Acanthamoeba* to a fresh plate (Fig. 2a), and the amoebae were cloned by dilution.

Acanthamoeba isolated in agar plates from nine clinical specimens and from eight samples obtained from four DWTPs (during winter and spring samplings) were then transferred into axenic cultures by placing the amoebae into PYG medium (0.75% proteose peptone, 0.75% yeast extract, and 1.5% glucose with 40 μg gentamicin per milliliter) at 28°C without shaking (Fig. 2b). Seventeen strains were established and characterized and eleven of them were inoculated in mice to assess their pathogenic profile (Table 2).

Molecular methods

DNA extraction DNA was extracted from 200 μl of the concentrated water samples and from the axenically established strains by using the *DNAeasy® Blood & Tissue* (QUIAGEN, Valencia, Calif.) and following manufacturer's instructions.

Real-time PCR assay A real-time PCR was used for the detection of *Acanthamoeba* (Qvarnstrom et al. 2006). Real-time PCR reactions were performed following the cycling structure and conditions described elsewhere (Qvarnstrom et al. 2006).

Sequencing Forward and reverse primers *JDP1* (5′-GGCCCAGATCGTTTACCGTGAA) and *JDP2* (5′-TCTCACAAGCTGCTAGGGGAGTCA) which amplify a fragment of approximately 500 bp of the ASA. S1 gene was used to produce amplicons of the isolates for genotyping

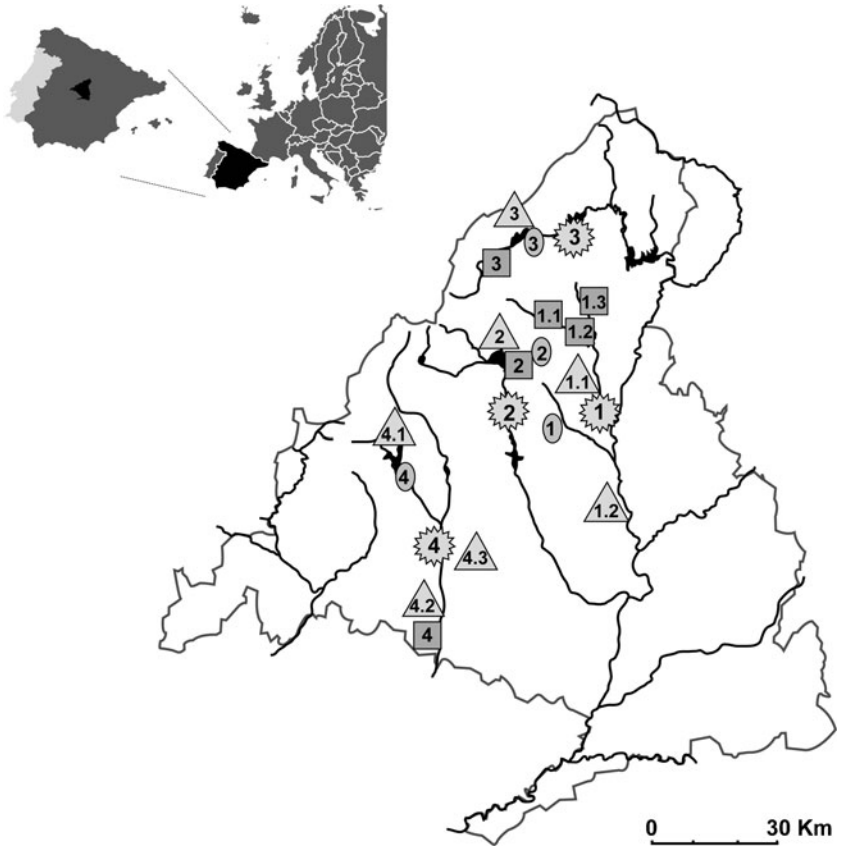

Fig. 1 Geographical location of the studied sampling points. Four Drinking Water Treatment Plant (DWTPs) ◯: Colmenar (*1*), Santillana (*2*), Pinilla (*3*), and Valmayor (*4*); seven Waste Water Treatment Plant (WWTPs) △: Guadalix (*1.1*), Arroyo de la Vega (*1.2*), Santillana (*2*), Pinilla (*3*), Los Escoriales (*4.1*), Navalcarnero (*4.2*), and Arroyo del Soto (*4.3*); and six Locations of Influence (LIs) □: Guadalix gauging station (*1.1*), Pedrezuela reservoir (*1.2*), Gargera gauging station (*1.3*), Santillana reservoir (*2*), and Lozoya gauging station (*3*), Guadarrama edge of river (*4*); located on four river basins ✹: Guadalix/Jarama (*1*), Manzanares (*2*), Lozoya (*3*), and Guadarrama (*4*) were studied

purposes as described previously (Schroeder et al. 2001). The PCR product was purified with the *Strata Prep PCR purification kit*® (Stratagen, La Jolla, CA, USA) and sequenced on both ends through cycle sequencing reactions using the Big dye V3.1 chemistry (ABI, Foster City, CA, USA). The sequencing reactions were purified in the Centri-sep columns® (Princeton Separations, Adelphia, NJ, USA) and loaded onto the ABI 3130x1 Genetic Analyzer® (Applied Biosystems, Foster City, CA, USA). Assembling, editing, and aligning of the sequences were done with GeneStudio suite (GeneStudio Inc., Swanee, GA) and with DNASTAR Lasergene 7 SeqMan (DNASTAR Inc., Madison, WI). To determine the genotypes, sequencing data was aligned with *Acanthamoeba* genotype sequences available in GenBank using the SeqMan program.

Table 1 Origin of the samples studied

Sample type	Source	*n*
Clinical	Corneal scrapes	26
Clinical	Contact lens solution (Madrid)	5
Clinical	Contact lens (Madrid)	9
Clinical	Vitreous humor (Madrid)	4
Environmental	DWTP ws	16
Environmental	DWTP ss	16
Environmental	WWTP ss	28
Environmental	LI ss	12
		Total, 116

DWTP drinking water treatment plant, *WWTP* wastewater treatment plant, *LI* location of Influence, *ws* winter sampling, *ss* spring sampling

Pathogenicity testing

Thirty six 6-week-old nude mice were divided into 12 groups of three animals and as previously described (Gianinazzi et al. 2005) were intranasally inoculated with 1×10^6 trophozoites/mouse in 6 μl of Page amoeba saline per strain, including a mock-infected group (negative control) that was inoculated with Page amoeba saline alone. The eight environmental strains were inoculated to evaluate their potential pathogenic properties in this model and three strains (USP-CR2-A32, USP-CR5-A35, and USP-CR6-A36)

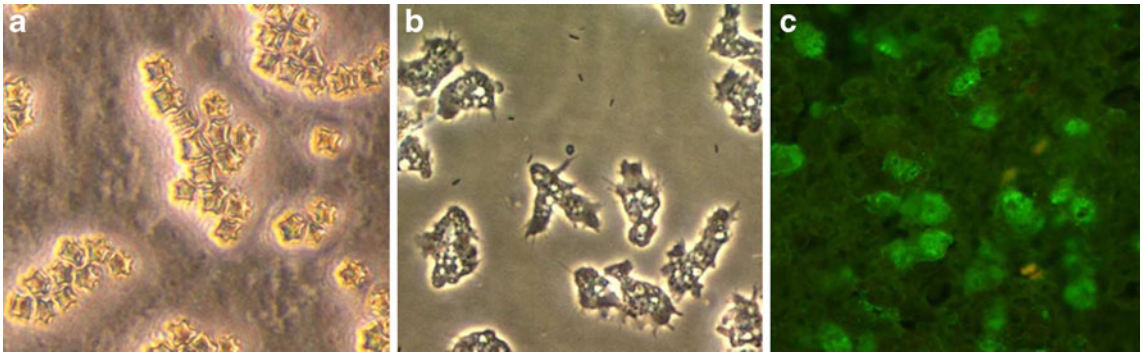

Fig. 2 **a** *Acanthamoeba* isolate from a water sample in agar plate showing the characteristic polyhedric cysts. **b** Trophozoites growing in PYG medium. **c** Brain tissue section of a nude infected mouse with the environmental strain USP-AWP-14 showing *Acanthamoeba* trophozoites by IFA, ×40 magnification

from AK patients were used as positive controls (Table 2). The mice were checked daily for clinical signs of CNS infection such as ruffling of fur, aimless wandering, and coma. Upon onset of clinical signs, the animals were sacrificed and tissue samples from brain and lungs were collected and used for (a) isolation of viable amoebae and (b) immunofluorescence studies.

Tissue samples from the brain and lung of all infected nude mice were gently disrupted and inoculated on agar plates as described above. The plates were sealed, kept at 34°C, and examined daily for the presence of amoebae.

Immunofluorescence identification of Acanthamoeba Immunofluorescence (IFA) was performed on deparaffinized brain and lung tissue sections as previously described (Seijo Martinez et al. 2000) except a 1:1,000 dilution of rabbit anti-*Acanthamoeba castellanii* polyclonal hyperimmune sera made in our laboratory was used.

Results

Acanthamoeba in water samples

The 16 water samples initially obtained from DWTP in winter were all positive for *Acanthamoeba* by real-time PCR (100%) and 15 of them (93.8%) by agar culture. This fact encouraged us to repeat the same sampling points (DWTP) in spring as well as to increase the sampling area including different kinds of water (WWTP and LI). In spring, we determined by real-time PCR the presence of *Acanthamoeba* spp. directly in 47 of the 56 spring samples

Table 2 Origin and genotypes of the investigated *Acanthamoeba* strains

Strain	Sequence type	Origin	Source of reference
USP-C3-A6	T4/9	Keratitis patient's cornea	New isolate
USP-C3-A7	T4/9	Keratitis patient's cornea	New isolate
USP-M-A8	T4/1	Keratitis patient's cornea	New isolate
USP-CR1-A31	T4/9	Keratitis patient's cornea	New isolate
USP-CR2-A32[a]	T4/9	Keratitis patient's cornea	New isolate
USP-CR3-A33	T4/8	Keratitis patient's cornea	New isolate
USP-CR4-A34	T4/9	Keratitis patient's cornea	New isolate
USP-CR5-A35[a]	T4/9	Keratitis patient's cornea	New isolate
USP-CR6-A36[a]	T4/9	Keratitis patient's cornea	New isolate
USP-AWP-A9[a]	T4/8	Finished water DWTP1 w1s	New isolate
USP-AWP-A10[a]	T4/8	Finished water DWTP2 w2s	New isolate
USP-AWP-A11[a]	T4/8	Finished water DWTP1 w2s	New isolate
USP-AWP-A12[a]	T4/8	Finished water DWTP2 w1s	New isolate
USP-AWP-A13[a]	T4/8	Finished water DWTP1 s1s	New isolate
USP-AWP-A14[a]	T4/8	Raw water DWTP1 w1s	New isolate
USP-AWP-A15[a]	T4/8	Raw water DWTP4 w1s	New isolate
USP-AWP-A16[a]	T4/8	Finished water DWTP3 w1s	New isolate

[a]Strain used for the pathogenicity test

DWTP1 drinking water treatment plant on river basin 1, *DWTP2* drinking water treatment plant on river basin 2, *DWTP3* drinking water treatment plant on river basin 3, *DWTP4* drinking water treatment plant on river basin 4, *w1s* first winter sampling, *w2s* second winter sampling, *s1s* first spring sampling, *s2s* second spring sampling

studied (83.9%), and in agar culture, we isolated *Acanthamoeba* spp. in 50 samples (89.3%) (Table 3). Considering all the samples (72) and the sampling points (28) analyzed in winter and spring, 57 of the samples were positive by both techniques while six and eight were positive either by real-time PCR or by culture, respectively (Tables 3 and 4). The only negative sample by both techniques was the raw water from DWTP-1 obtained in the second spring sampling meaning that *Acanthamoeba* sp. was detected at the entrance of 93.8% of DWTP but in the 100% of finished water (Tables 3 and 5). However, the water sample obtained from the same point in winter was positive. Finally, 27 of 28 sampling points (96.4%) were positive for *Acanthamoeba* spp. in spring, but all of them were positive considering all the time period analyzed.

Table 3 Presence of *Acanthamoeba* sp. in water samples from the spring sampling

	Origin	*Acanthamoeba* sp.	
		Agar culture (1 s/2 s)	Real-time PCR (1 s/2 s)
Winter	DWTP-1. R	+/+	+/+
	DWTP-1.F	+/+	+/+
	DWTP-2.R	+/+	+/+
	DWTP-2.F	+/+	+/+
	DWTP-3.R	+/+	+/+
	DWTP-3.F	+/+	+/+
	DWTP-4.R	+/+	+/+
	DWTP-4.F	−/+	+/+
	Total	93.8%	100%
Spring	DWTP-1. R	+/−	+/−
	DWTP-1.F	+/+	+/+
	WWTP-1.1.R	+/−	+/+
	WWTP-1.1.F	−/−	+/+
	LI-1.1	+/+	+/+
	WWTP-1.2.R	+/+	+/+
	WWTP 1.2 F	+/+	+/+
	LI-1.2	+/−	+/+
	LI-1.3	+/+	+/−
	DWTP-2.R	+/+	+/+
	DWTP-2.F	+/+	+/+
	WWTP-2.R	+/+	+/+
	WWTP-2.F	+/+	+/+
	LI-2	+/+	+/+
	DWTP-3.R	+/+	+/−
	DWTP-3.F	+/+	+/+
	WWTP-3.R	+/+	+/−
	WWTP-3.F	+/+	+/−
	LI-3	+/+	+/−
	DWTP-4.R	+/+	+/+
	DWTP-4.F	+/+	+/+
	WWTP-4.1.R	+/+	+/−
	WWTP-4.1.F	+/+	+/−
	WWTP-4.2.R	+/+	+/+
	WWTP-4.2.F	+/+	+/+
	WWTP-4.3.R	+/−	+/+
	WWTP-4.3.F	+/+	+/+
	LI-4	+/+	+/−
	Total	89.3%	83.9%

The points of sampling are grouped by fluvial basins

DWTP drinking water treatment plant, *WWTP* wastewater treatment plant, *LI* location of influence, *R* raw water, *F* finished water, *1 s/2 s* first sampling/second sampling

Molecular characterization of *Acanthamoeba* spp. isolates and strains

From the 63 positive samples by real-time PCR, only 24 could be amplified with the JDP primers. All of them were characterized as the T4 genotype group based on the sequencing analysis of this fragment. By applying the nomenclature of Booton et al. (2002), a total of 22 samples (91.7%) are grouped within the T4/8 genotype and two of them (8.3%) within T4/1 (Table 6).

Additionally, a collection of 8 *Acantamoeba* spp. strains obtained from water samples (winter and spring) from raw and finished water of four DWTPs were established in axenic liquid medium. In all cases, a T4/8 genotype was observed (Tables 2 and 6). Of the nine clinical strains, seven (78%) were genotype T4/9, one (11%) was T4/8, and one (11%) was T4/1 (Tables 2 and 6).

Pathogenic potential in nude mouse model of environmental *Acanthamoeba* T4 strains

Experimental infection of nude mice with the eight environmental *Acanthamoeba* T4 strains resulted in the development of clinical signs characteristic of free-living amoeba CNS

Table 4 Comparison between results obtained by real-time PCR and by agar culture with raw and finished water of the winter and spring samples

Sampling point	Real-time PCR		Culture	
	No.+S/TS	%	No.+S/TS	%
DWTP	30/32	93.8	30/32	93.8
WWTP	24/28	85.7	24/28	85.7
LI	9/12	75.0	11/12	91.7
Total	63/72	87.5	65/72	90.3

No.+S number of positive samples, *TS* total samples, *%* percentage, *DWTP* drinking water treatment plant, *WWTP* wastewater treatment plant, *LI* location of influence

Table 5 Comparison between results obtained by real-time PCR and by culture with raw or finished water of the winter and spring samples

Sampling point	DWTP				WWTP			
	Culture		Real-time PCR		Culture		Real-time PCR	
	No.+S/TS	%	No.+S/TS	%	No.+S/TS	%	No.+S/TS	%
Raw water	15/16	93.8	14/16	87.5	12/14	85.7	12/14	85.7
Finish water	15/16	93.8	16/16	100	12/14	85.7	12/14	85.7
Total	30/32	93.8	30/32	93.8	24/28	85.7	24/28	85.7

No.+S number of positive samples, *TS* total samples, *%* percentage, *DWTP* drinking water treatment plant, *WWTP* wastewater treatment plant, *LI* location of influence

infection such as reduced level of alertness or consciousness and ataxia. The animals were sacrificed 24–48 h after the onset of the signs. After day 9 post-infection (p.i.), all animals either died or were sacrificed because they presented symptoms of CNS infection. The nine mice considered as positive controls and inoculated with the human strains USP-CR2-32, USP-CR5-35, and USP-CR6-36 were sacrificed at day 5 p.i.; and the three mice inoculated with Page amoeba solution and considered as negative controls were sacrificed at day 28. The examination of IFA-stained brain and lung tissue sections from all, excepting the negative control mice, showed multiple organisms invading both tissues, displaying areas marked by a dense presence of trophozoites (Fig. 2c). Re-isolation of throphozoites was successful for the brain and lungs of all infected animals with the environmental or the human *Acanthamoeba* isolates. In the three mice used as the negative control, no clinical signs of infection became apparent and amoebae were not isolated from tissues samples at any time.

Table 6 Genotypes from environmental and human strains and isolates characterized

	Origin	Genotype
Winter samples	DWTP-1. R 1s	T4/8
	DWTP-1. F 1s	T4/8
	DWTP-1. F 2s	T4/8
	DWTP-2. F 1s	T4/8
	DWTP-2. F 2s	T4/8
	DWTP3. F 1s	T4/8
	DWTP-4. R 1s	T4/8
Spring samples	DWTP-1. F 1s	T4/8
	DWTP-1. F 2s	T4/8
	WWTP-1.2. R 2s	T4/8
	WWTP-1.2. F 2s	T4/1
	LI-1.2. 2s	T4/8
	DWTP-2. R 2s	T4/8
	LI-2. 2s	T4/8
	DWTP-3. F 2s	T4/8
	WWTP-3. F 1s	T4/8
	LI-3. 2s	T4/8
	DWTP-4. F 1s	T4/8
	DWTP-4. F 2s	T4/8
	WWTP-4.1. F 1s	T4/8
	WWTP-4.1. F 2s	T4/8
	WWTP-4.2. R 2s	T4/8
	WWTP-4.2. F 2s	T4/8
	WWTP-4.3. F 2s	T4/1

DWTP: Drinking water treatment plant; WWTP: Wastewater treatment plant; LI: Location of influence, R: Raw water; F: Finished water. AK: *Acanthamoeba* keratitis. 1 s: 1st sampling, 2 s: 2nd sampling.

Discussion

Acanthamoeba spp. has come under increased attention recently because of many recognized outbreaks of *Acanthamoeba* keratitis (Acharya et al. 2007; Verani et al. 2009). Since they are commonly found in various environmental water sources throughout the world (Schuster and Visvesvara 2004), an increasing interest in a better quality control of water sources for human use has pointed out the necessity to carry out epidemiological studies based on molecular tools to better define the risk factors and sources of infection of these *Amoebae*.

A wide variety of sampling ranging from 5 to 100 l of water and concentration methods including filtration and other devices for *Acanthamoeba* retention and elution have been proposed (Lorenzo-Morales et al. 2005b; Bonilla-Lemus et al. 2009; Gianinazzi et al. 2009; Valster et al. 2009; Boost et al. 2008; Thomas et al. 2008) making the results obtained difficult to compare. We therefore decided to use the IDEXX® Filta-Max system to concentrate *Acanthamoeba* since this system has already been validated for the detection of *Giardia* and *Cryptosporidium* by the US EPA. Using this system would not only allow the use of the same sample of water for the detection and enumeration of *Acanthamoeba* as well as *Giardia* and *Cryptosporidium* but also would save time and cost.

From the 72 water samples studied, we observed 87.5% and 90.3% of *Acanthamoeba*-positive samples by real-time PCR or agar culture, respectively. If we analyze the different types of water studied, *Acanthamoeba* spp. was detected by real-time PCR and culture in 93.8% of DWTP samples, in 85.7% of WWTP samples, and in 75% and 91.7% of LI samples. This high presence of *Acanthamoeba* in water samples from the central area of Spain correlates with the limited data available in the literature and with the fact that a high seroprevalence (>80%) of anti-*Acanthamoeba* antibodies has been observed in healthy population (Chappell et al. 2001; Brindley et al. 2009) suggesting a potential high environmental contamination. Although a number of studies carried out in many different regions (except the central area) of Spain have identified *Acanthamoeba* spp. in rivers (Marti Mallen et al. 1986), bottled mineral water (Varela Mato et al. 1987), public fountains (Paniagua Crespo et al. 1990), thermal spas (Penas Ares et al. 1994), aqua cultures of turbot mussels (Lloves et al. 1996), tap water and sea water (Lorenzo-Morales et al. 2005b), and beach sand (Lorenzo-Morales et al. 2005a), there is only one report of *Acanthamoeba* spp. identification from Central Spain in drinking water fountains (Madrigal Sesma et al. 1982). However, the present study is the only one that identifies the presence of *Acanthamoeba* in raw and finished water from DWTP and WWTP originating from the Central Spain.

Similarly, *Acanthamoeba* spp. has been identified in many countries around the world for example in Bulgaria (Tsvetkova et al. 2004), Osaka (Japan) (Edagawa et al. 2009), USA (John and Howard 1995; Ettinger et al. 2003; Shoff et al. 2008; Stockman et al. 2011; Mahmoudi et al. 2012), Taiwan (Hsu et al. 2009), Brazil (Caumo et al. 2009; Magliano et al. 2009), South Korea (Jeong and Yu 2005), and Switzerland (Gianinazzi et al. 2009).

In this study, raw and finished water from DWTP and WWTP has been analyzed by real-time PCR and agar cultivation in order to assess the efficacy of disinfection methods used. Although real-time PCR is a more sensitive method, it can be inhibited by many substances in the sample, and this is a particularly major problem when testing environmental samples. Therefore, we decided to use both methods. The culture method seemed to be slightly more sensitive than the real-time PCR (90.3% versus 87.5%). However, it is important to bear in mind that the number of samples analyzed was not enough to show significant differences. The only difference between the two methods was seen in the sample LI (91.7% versus 75.0%) suggesting that the characteristics of the water sample may have influence on the sensitivity of the method used (Table 4). The data show that the 28 sampling points (100%) should be considered as positive for *Acanthamoeba* spp., indicating that the central area of Spain bears the burden of one of the highest rates of the presence of *Acanthamoeba* in water samples reported to date. Moreover, *Acanthamoeba* spp. was detected in the raw and finished water of all DWTPs and WWTPs, indicating that the purification processes used in these treatment plants did not eliminate this opportunistic agent. This fact is important to highlight since such amoebae have been described as vectors or reservoirs of pathogenic microorganisms (Scheid et al. 2008; Valster et al. 2011; Huang et al. 2011). Our results are not surprising, since similar data have been obtained by the previous reports from DWTP. In France, Thomas et al. (2008) in a study carried out in a DWTP with Seine River water showed that FLA are resistant to the treatment chain of sand filtration, ozonation, carbon filtration, and chlorination. Similarly in Germany, Hoffman and Michel (2001) demonstrated that FLA could survive the water purification processes used. Finally, Edagawa et al. recognized that in Osaka (Japan), the same sequence of purification process was not able to decontaminate drinking water from FLA (Edagawa et al. 2009). However, to our knowledge, this is the first study in the characterization of potentially pathogenic *Acanthamoeba* from WWTP sewage effluents.

Based on the molecular characterization, all environmental *Acanthamoeba* isolates used in this study have been identified as T4 genotype. The clinical samples were also identified as T4 genotype, but most of them (78%) were T4/9 and the others T4/8 (11%) and T4/1 (11%). According to many studies carried out so far (Booton et al. 2005; Booton et al. 2002; Ledee et al. 2009), genotype T4 is not only the most frequent genotype causing AK in humans, but it is also the most frequently isolated from environmental samples. With the worldwide prevalence of genotype T4, regardless of region, it is not surprising that about 90% of *Acanthamoeba* isolates associated with AK are genotype T4 (Ledee et al. 2009).

In most of the previous studies on *Acanthamoeba* epidemiology carried out in Spain, specific characterization was made based on morphological features. In Galicia (NW, Spain), in most cases, *Acanthamoeba polyphaga* was reported; *A. polyphaga* belongs to T4 genotype. Additionally, *Acanthamoeba griffini* and *Acanthamoeba astronyxis* were also reported, so T3 and T7 would also be present in this region of Spain (Arias Fernandez et al. 1989; Lloves et al. 1996; Paniagua Crespo et al. 1990). Although, molecular techniques were applied to the studies carried out on isolates from tap and sea water in the Canary Islands, the authors identified the isolates as *A. polyphaga*, *A. astronyxis*, or *Acanthamoeba* spp. (Lorenzo-Morales et al. 2005b; Lorenzo-Morales et al. 2005a). With this information, we may only assume that T4 and T7 genotypes are represented in the Tenerife environmental samples. Data from countries such as Brazil (Magliano et al. 2009), France (Thomas et al. 2008), Switzerland (Gianinazzi et al. 2009), South Korea (Jeong et al. 2007), and USA (Booton et al. 2004) have provided supporting evidence for that. However, in Osaka (Japan) (Hsu et al. 2009), the most frequent genotype was T3 (56.8%) and T4 (13.5%); and in Taiwan (Edagawa et al.

2009), *A. griffini* (T3) was the most frequent followed by *Acanthamoeba jacobsi* (T15).

A comparison of the genotypes from strains of clinical origin from this study with those from other studies that investigated multiple AK isolates revealed that our study had the T4/1 and T4/9 in common with France where T4/2 and T4/13 (Yera et al. 2008) were also seen. In Italy, 78% of cases were T4 including T4/1 and T4/9 and 21% were T3 (Di Cave et al. 2009), similarly in China, 94% of AK cases were associated to T4 (T4/1 and T4/9) and to T3 genotypes (6%) (Zhang et al. 2004). However, in USA, although 97.2% of cases were T4, the T4/1 and T4/9 were not represented (Ledee et al. 2009). In Spain, an AK isolate was reported as T4 (Lorenzo-Morales et al. 2007).

The dominance of T4 genotype in the environmental and clinical isolates characterized in this study correlates with the worldwide dominance of this genotype in environmental and AK isolates (Booton et al. 2005; Ledee et al. 2009). Although genotypes found in Spanish clinical strains (T4/1 and T4/9) were comparable to the isolates from other countries, they had a low representation in Spanish environmental isolates (T4/1=8.3%, T4/9=0%). For this reason, we decided to investigate the pathogenic potential of the Spanish strains obtained from finished water of DWTPs, with genotype (T4/8); that was, however, underrepresented in the Spanish clinical strains (11%). The data from the experimental in vivo study suggest that the environmental isolates might be potentially pathogenic to humans with a reduced or impaired immune status because of malnutrition, diabetes, pregnancy, alcoholism, immunosuppressive therapy, and AIDS; all of them considered as risk factors for *Acanthamoeba* infection (Khan 2006). Known risk factor for AK such as poor contact lens hygiene and behaviors such as swimming or showering with contact lenses (Joslin et al. 2007) highlights the importance of the presence of *Acanthamoeba* in domestic water supply. In conclusion, we show for the first time that *Acanthamoeba* T4 genotype is present in a very high percentage (100%) in different types of water samples including tap water and sewage effluents in the central area of Spain suggesting a potential health threat for humans especially for the contact lens wearers.

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