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Research paper

Molecular and morphological characterization of *Acanthamoeba* isolated from corneal scrapes and contact lens wearers in Argentina

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ABSTRACT

In this study, we describe the frequency of *Acanthamoeba* keratitis (AK) in patients that assisted in the Ophthalmology Department and determine the species/genotypes of free living amoebas (FLA) isolates. FLA from Corneal scrapes (CS) and contact lens (CL) wearers were studied by morphological and molecular characterization. A database was constructed with sociodemographic, clinical findings and history of use of CL variables. During January 2000 and September 2016 patients with corneal pathology admitted to the Ophthalmology Service of the University Hospital in Córdoba city, Argentina were included in the study. FLA were detected in 1.5% (11/739) and in 17% (11/65) of CS and CL analyzed respectively. FLA isolates from CL users evidenced an 80.9% of inappropriate lens maintenance, 4.8% (1/21) were not CL users that have been in contact with waters in outdoor environment and 14,3% (3/21) with no data about CL users. *Acanthamoeba* was confirmed in 100% and 82% of CS and LC respectively. The most frequent symptom associated with AK was red eye and photophobia. FLA from CS belonged to group II but 82% (9/11) and 18% (2/11) from CL belonged to group II and III respectively. T4 genotype and *A. polyphaga* species were detected in 100% of *Acanthamoeba* isolates. Poor CL hygiene practices, highlights the need for improved education about the severity of AK and consequences of improper CL hygiene. Genotype T4 detected in 100% of both CS and CL samples, consistently with previous findings indicating that this genotype is by far the most prevalent isolated from ocular infection.

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1. Introduction

The free-living amoebas (FLA) are ubiquitous microorganisms representing one of the most prevalent protozoans found in the environment (Mahmoudi et al., 2012; Costa et al., 2010). FLA are frequently found in natural and artificial niches (soil, air, freshwater, swimming pools, tap water and ocean sediments) but under certain conditions may play a role as opportunistic pathogenic microorganisms. Among FLA, species of the genus *Acanthamoeba* have adapted to live in a variety of human tissues, where two main pathologies: the granulomatous amoebic encephalitis (GAE) in immunocompromised individuals and *Acanthamoeba* keratitis (AK) are well documented (Khan, 2006; Lorenzo-Morales et al., 2013; Visvesvara, 2010; Clarke et al., 2012; Lorenzo-Morales et al., 2015). About 20 species of *Acanthamoeba* have been considered and described in groups I, II, and III according to cyst size, the shape of the inner (endocyst) and outer wall (ectocyst) (Pussard and Pons, 1977; Page, 1976), but other criteria like tolerance to temperatures and high osmolarity, or cytopathic effects on cultured cells have been considered to define not only differences but also pathogenic potential of many species (Lorenzo-Morales et al., 2015).

Molecular studies focusing on the sequences of the small-subunit 18S rRNA genes are currently the main tool for taxonomic characterization of *Acanthamoeba*. A total of 17 genotypes has been reported, whereas genotype T4 is the most frequently involved in cases of AK (Stothard et al., 1998; Walochnik et al., 2000; Booton et al., 2009; Corsaro and Venditti, 2010; Nuprasert et al., 2010). For example, >90% of AK cases have been linked with this genotype. Similarly, T4 has been the major genotype associated with the non-keratitis infections such as AGE and cutaneous infections. At present, it is unclear why T4 isolates are most abundant in human infections but it is likely due to their greater virulence and properties that enhance their transmissibility as well as their reduced susceptibility to chemotherapeutic agents. AK is a painful and progressive infection of the cornea occurring in individuals with normal immunological status. If the infection is not diagnosed at time and aggressively treated, corneal transplantation might be required (Jiang et al., 2015). The number of AK cases diagnosed has increased

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dramatically over the last 20 years and incidence of AK in recent decades is attributed to the popularization of contact lenses but especially in those users with poor hygiene practices or by using cleaning and disinfection procedures with tap water or homemade saline solutions. This explains why contact lens users comprise >85% of AK patients (Radford et al., 2002; Dart et al., 2009; Ibrahim et al., 2007; Page and Mathers, 2013). Nevertheless, other physical agents, such as mud can also cause corneal injury and carry amoebae to the cornea (Bouheraoua et al., 2013).

Worldwide, AK is still considered a rare disease with an estimated prevalence that depends on the populations studied (1–9/100,000). In South America, few reports describe local incidence of this pathology (Duarte et al., 2013). In Argentina, only isolated cases of this pathology have been reported (Gertiser et al., 2010; Menghi et al., 2012), whereby the lack of AK incidence data is evident. Therefore, the aim of this study was to determine the frequency of AK in patients that assisted in the Ophthalmology Department of the Cordoba University Hospital and to conduct morphological and molecular characterization of *Acanthamoeba* isolates obtained from these patients.

2. Materials and methods

2.1. Samples, FLA isolation, growth and morphological characterization

During January 2000 and September 2016, corneal scrapes (CS, $n = 739$) and contact lenses (CL, $n = 65$) from patients with corneal pathology admitted to the Ophthalmology Service of the University Hospital in Córdoba city, Argentina were included in the study. Clinical symptoms at the time of medical consultation, the use of contact lenses and cleaning procedures, their use when bathing in pools or rivers and previous antimicrobial treatments were recorded in patients from 2011–2016 prior samples collection. Then, after a comprehensive slit-lamp examination, 0.1% propamidine eye drops like topical anesthesia were administered and specimens were collected by scraping the corneal lesion at its peripheral borders using a 25 G needle. Immediately, samples were placed onto 1.5% non-nutritive saline PAGE's solution agar plates seeded with *Escherichia coli* ATCC 25922 (ANNE) (Garcia, 2010). An additional sample in a similar volume was taken from the remaining unscraped peripheral border of the lesions and collected into a sterile Eppendorf tube with 2 mL of PAGE solution, resuspended using a vortex shaker for 30 s for tissue release, centrifuged 1 min at 2000 rpm and subsequently pellets were examined. Contact lens rising liquids (CL) from lens boxes were trespassed to sterile tubes, centrifuged 5 min at 1000 rpm and pellets were seeded onto ANNE plate prior being examined by light microscopy. ANNE plates with corneal and/or LC samples were sealed, incubated at 30 °C for 7 days and monitored daily by examination under inverted microscope. The presence of FLA was confirmed based on the observation of trophozoites and cysts harvested from culture plates by washing them with 2 mL PAGE solution under laminar air flow hood. After 2 days culture, when trophozoites were evident, they were resuspended with 0,7% Trypan blue solution and observed at light or phase contrast microscopy to evaluate amoeba viability and for the presence of *Acanthamoeba* ancanthopodia. Cysts were measured by using an ocular micrometer scale under a light optical microscope and morphological characteristics were classified according to groups I, II, and III based on cyst size and shape, following previous descriptions (Khan, 2006; Visvesvara, 2010; Pussard and Pons, 1977).

2.2. DNA extraction and molecular characterization

Amoebae cysts were harvested from 5 days old cultures by rinsing agar surfaces with sterile PAGE solution and adjusted to 10^4 cysts/mL by dilution using Neubauer counting chamber. Total DNA was extracted by using Accuprep Stool DNA Extraction Kit (Bioneer, Korea) following the manufacturer's protocol and DNA concentration was determined using a Nano Drop 1000 spectrophotometer (Fisher Scientific). DNA was subjected to PCR aiming at the specific recognition of 18S rDNA from amoeba of the genus *Acanthamoeba* using the primers JDP1 (5′-GGC CCA GAT CGT TTA CCG TGA A-3′) and JDP2 (5′-TCT CAC AAG CTG CTA GGG GAG TCA-3′) which amplify a region ASA.S1 of approximately 500 bp (Gertiser et al., 2010; Menghi et al., 2012; Garcia, 2010; Schroeder et al., 2001). Amplifications were carried out in a 50 μL volume containing 2–5 ng of DNA, 0.2 μM dNTPs mix (GE Healthcare, Buckinghamshire, England, UK), 1 μM of each oligonucleotide, reaction buffer (50 mM KCl2, 10 mM Tris–HCl), 1.5 mM magnesium chloride, and 1,5 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). A DNA thermal cycler was used for 42 cycles as follows: 95 °C for 7 min, then 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, 40 cycles of 72 °C for 10 min. In control experiments, each PCR was conducted with a template DNA-free blank. DNA from a reference strain *A. polyphaga* (ATCC 30461) as positive control, distilled water (instead of DNA) and purified *Blastocystis* sp. DNA were added to the reaction mixture as the negative controls. Aliquots of 10 μL from each PCR reaction (diluted 1/10) was subjected to a 1% agarose gel electrophoresis, stained with 0.1 lM/mL ethidium bromide and observed under a UV-light transilluminator. DNA sequencing was based on standard Sanger Dideoxynucleotide method (Macrogen, Korea) and sequences were compared by using the Basic Local Alignment Search Tool (BLASTn) program of the US National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) to classify our *Acanthamoeba* isolates

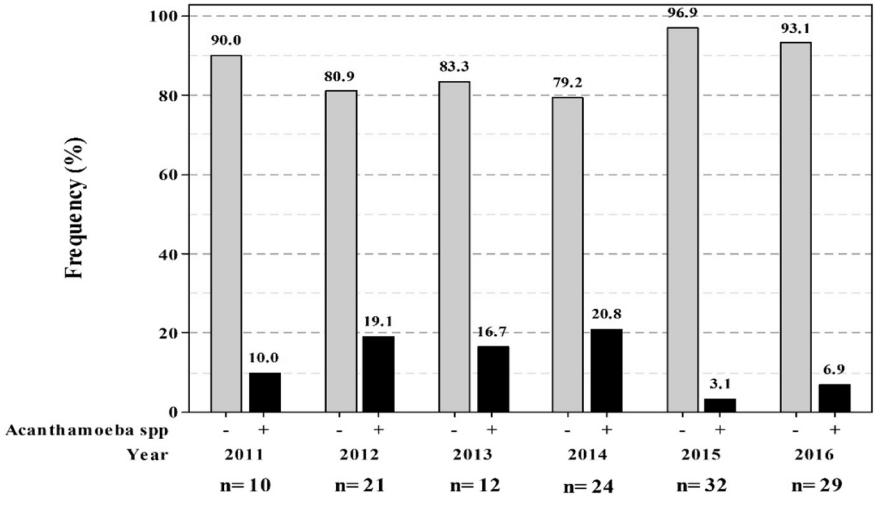

Fig. 1. Temporal variation of *Acanthamoeba* positive cultures.

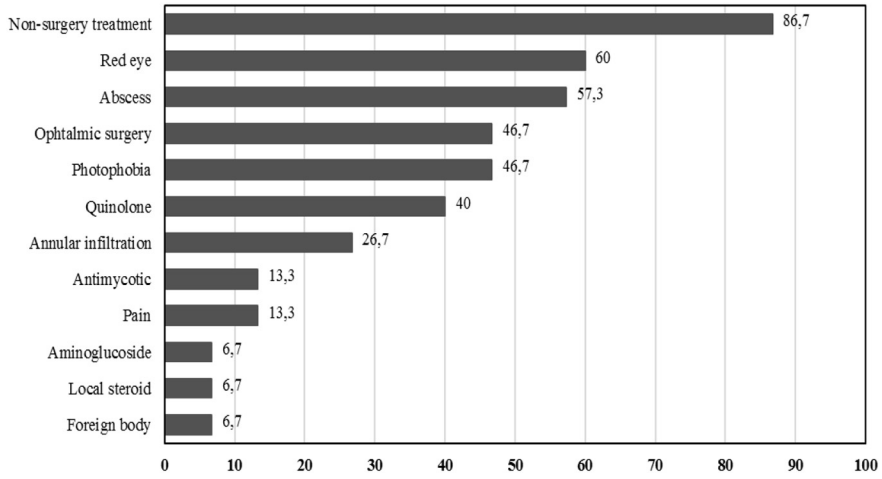

Fig. 2. Frequency of some characteristics present in patients with positive cultures for *Acanthamoeba* spp.

into the different genotypes. Sequences derived from the amplicons obtained with primers JDP1 and JDP2 were edited by using the bioinformatics tool MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets (Kumar et al., 2016). They were then subjected to a homology analysis with sequences available in the GenBank database using the NCBI BLASTn bioinformatic tool (NCBI, USA), discarding two sequences with no results of homology (Hits). Additionally, the nucleotide sequences of all genotypes of *Acanthamoeba* spp. were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/pubmed). The sequences of the present study and reference strains were analyzed by MEGA 7.0 program, constructing alignments thereof by Muscle tool, implemented in MEGA. The phylogenetic tree was reconstructed using Kimura two-parameter distance algorithm with 1000 replicates using neighbor-joining method. The sequence of strain *Acanthamoeba* spp. V006, T1 genotype, was used to root the final tree.

2.3. Statistical analyses

A database was constructed in Excel® format, with sociodemographic variables (date of care, age, sex, occupation) and clinics, among which the following were included: 1. Etiology of the clinical picture (infection, trauma, foreign body), 2. Symptoms (pain, red eye, photophobia), 3. Clinical findings (ulcer, ring infiltration, abscess), 4. History of ocular surgery or topical medical management (group of antibiotics, eye drops, steroids or biguanide polyhexamethylene (PHMB)); All the mentioned clinical variables were dichotomized. We also collected information about the history of use of contact lenses and characteristics of use, including: time of use, bath with lenses and frequency of cleaning, which was grouped according to the level of "cleaning per month" (>1, 1, <1, never). The statistical packages Minitab®-V17 and Epi-Info Version 3.01 were used to analyze the data. A descriptive analysis was performed expressing the data in proportions and medians (25th–75th percentiles, IQR). The comparisons between continuous variables were performed with the U Mann Whitney test, and between categorical variables with Chi square; A p value < 0.05 was established as significant. In an univariate analysis, the magnitude of the association (crude OR, 95% CI) was determined between the presence of a positive culture for *Acanthamoeba* spp. and variables that demonstrate a significant relationship.

3. Results

3.1. Acanthamoeba frequency of infection

80.9% (17/21) of FLA isolates were recovered from CL users which had a history of inappropriate lens maintenance, 4.8% (1/21) not CL users that have been in contact with waters in outdoor environment and 14, 3% (3/21) with no data about CL users. Among CL users who had completed a questionnaire, there was 83% who were disinfecting irregularly and 67% who were swimming with CL *in situ*. Therefore, the

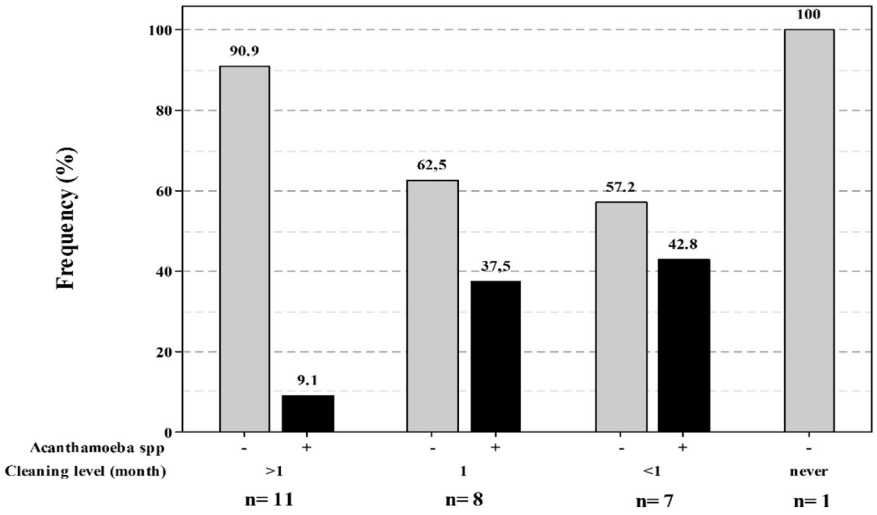

Fig. 3. Patients who used contact lenses, classified by cleaning frequency for one month and by positivity for *Acanthamoeba* spp.

Table 1
Characteristics retrieved from the clinical history of the patients and related to positive cultures of *Acanthamoeba*.

Variable	*Acanthamoeba* spp.		χ^2	df	*p*-value	Crude OR (95%, CI)
	Positive, *n* (%)	Negative, *n* (%)				
Contact lens						
Yes	7 (46.7)	21 (18.6)	6.111	1	0.013	3.83
No	8 (53.3)	92 (81.4)				(1.25–11.7)
PHMB						
Yes	4 (26.7)	7 (6.2)	7.065	1	0.008	5.50
No	11 (73.3)	106 (93.8)				(1.39–21.81)
Corneal ulceration						
Yes	– (0)	55 (48.7)	12.802	1	0.000	0 (0.0–0.31)
No	15 (100)	58 (51.3)				

most common risk factors were irregular or omitted disinfection and poor basic CL storage case hygiene. One or more of these risk factors were identified for 91% of these patients. Questionnaires also revealed that 100% of patients from whom *Acanthamoeba* was isolated, had one or more acute symptoms like photophobia, ocular pain, red eye or stromal infiltrates.

3.2. Descriptive analysis of socio-economic and epidemiological data

In the period between 2011–2016, 128 eye cultures were requested on suspicion of *Acanthamoeba* spp., with a positivity of 11.7% (95% CI, 5.8–17.7%), which in the first four years of follow-up ranged from 10–20.8%, while in the latter two was <7% (Fig. 1). The median age (p25–p75) was 41 years, without finding differences when comparing patients with positive or negative cultures. Results showed more infection rate in women (66.7%). Data on occupation were obtained in 59.4% of the patients (76/128), most were housewives, engaged in masonry/construction/painting or worked in various trades; In 8 of the 15 positives, 3 were traders, 2 housewives, 2 employees and doctor/biochemist; no relationship was found between the work performed with the positivity for *Acanthamoeba* spp. Fig. 2 shows the frequency of some characteristics present in patients with positive cultures for *Acanthamoeba* spp.; The majority required medical management and the most frequent treatment was the use of eyes drops with quinolones until *Acanthamoeba* diagnosis was confirmed. The most frequent symptom was red eye, followed by photophobia; Abscess was the most frequent pathological finding. About half of the patients reported having required some type of ophthalmic surgery. Fig. 3 presents the patients who used contact lenses, classified by cleaning frequency for one month and by positivity for *Acanthamoeba* spp. A tendency to decrease the positivity is evidenced, while the frequency of cleaning of lenses increases; only one patient was identified who, after 7 months of wearing contact lenses reported not having performed any type of cleaning. It should be noted that none of the characteristics presented in Figs. 2 and 3 presented statistical association with positive cultures for *Acanthamoeba* spp. Table 1 shows three clinical characteristics that showed a relationship with the positivity for *Acanthamoeba* spp. Among them, patients who used CL and those who received prior treatment with PHMB were more at risk of having positive cultures. The finding of corneal ulcer during physical examination was not present in patients with positive cultures. Finally, it was found that patients with positive cultures received a greater number of different treatments (median 2.5, RIQ 2.25) than patients with negative cultures (median 1, RIQ 2), with statistical differences between both groups (Mann-Whitney *U* test, P: 0.023, data not shown).

3.3. FLA isolation, growth and morphological characterization

In this study, ANNE cultures could detect the presence of FLA in 1.5% (11/739) and in 17% (11/65) of CS and CL analyzed respectively. On assay, 13 isolates, CS ($n = 8$) and CL ($n = 5$), were included for morphological and molecular characterization. FLA recovered form corneal cultures varied in size from 10.5 to 15 μm and showed globular or polyhedral endocysts, wavy ectocysts and morphological characteristics mainly to group II (Pussard and Pons, 1977; Duarte et al., 2013). Amoebae isolates from CL demonstrated phenotypic differences, where 82% (9/11) belonged to group II, with two-layer membrane: the outer wall moderately ondulated (exocyst) and endocyst wall typically polygonal arrangement that join together at the pore zone and 18% (2/11) to group III, showing round shapes with endocyst that does not join with exocyst (Fig. 4). Trophozoites from group II presented typical contractile vacuoles and filiform pseudopodia (acanthopodia), not detected in those belonging to group III.

3.4. Molecular characterization of Acanthamoeba species

By using a genus specific primer pair, ASA-S1 amplicon, *Acanthamoeba* was confirmed in 100% and 82% of CS and LC cultures respectively. Two AVL strains from LC (103 and 65) were phenotypically characterized in group III, where CL 65 did not amplify for amplicon

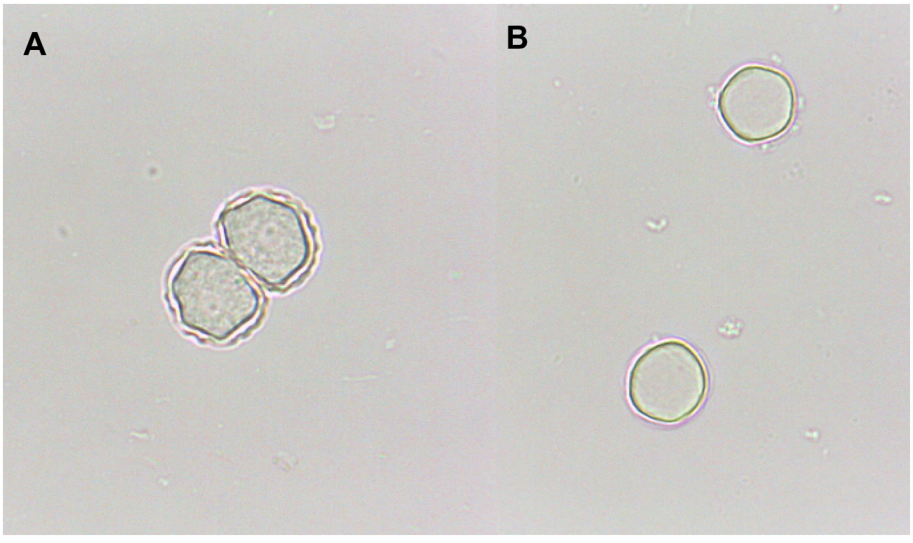

Fig. 4. Predominant cysts morphotypes observed after 7 days old cultures. A. Morphotype II observed in all isolates, except 65. B. Morphotype III in isolate 65. Light microscopy ×400. Bar: 10 μm.

Table 2
Pheno-genotypic characterization of AVL isolates from corneal scrapes and contact lens samples.

	Isolates												
	01	04	14	82	88	103	110	3	7	25	48	65	79
CS	+	+	+	+	+	+	+	−	−	−	−	−	+
CL	ND	−	ND	ND	ND	ND	ND	+	+	+	+	+	+
Predominant morphotype	II	II	II	II	II	III	II	II	II	II	II	III	II
PCR amplicon ASA.S1	+	+	+	+	+	+	+	+	+	+	+	NEG	+
Subtype	T4	T4	T4	T4	T4	T4	NR	T4	T4	T4	ND	ND	T4
Genotype *A. polyphaga*	+	+	+	+	+	+	ND	+	+	+	ND	ND	+

CS: corneal scrapings, CL: contact lens, (+): recovered, (−): unrecovered, ND: not done.

ASA S1, not belonging to *Acanthamoeba* genus. Sequence analysis of ASA-S1 amplicons showed T4 genotype in 100% of isolates assayed (Table 2) and phylogenetic reconstruction demonstrated 100% were related with *A. polyphaga* species (Fig. 5).

4. Discussion

AK has been worldwide a source of concern in recent decades because of the increasing number of reported cases, generally associated with contact lens users and in any case of corneal trauma with exposure to soil or contaminated water (Carvalho et al., 2009; Siddiqui and Khan, 2012; Carnt and Stapleton, 2016). Nevertheless, in South America few reports demonstrate the incidence of this pathology in patients suffering acute ocular disease (Duarte et al., 2013; Gertiser et al., 2010). To our knowledge this report as far presents the first that describes the AK frequency and morphological and molecular description of FLA isolates from ocular samples in Argentina. Our 15 years study revealed an AK frequency of 1.5% with an overall incidence estimated in 0.7/100,000. Nevertheless, for diagnostic and treatment purposes, the identification of the agent to genus is sufficient but for epidemiological studies is not. However, it is necessary to identify and characterize species to determine whether there are types more likely to be parasitic and to establish their probable niches.

In the present study 100% of isolates of *Acanthamoeba* obtained from cornea were classified in group II, in agreement with previous reports that correlates group II, as the one that harbors most of FLA pathogenic clinical isolates (Walochnik et al., 2015; Risler et al., 2013). Sequencing a partial SSU-rDNA region of 13 isolates demonstrated genotype T4 in 100% of both keratitis and CL samples, consistently with previous findings that indicate that this genotype is by far the most prevalent in samples from ocular infection, strengthening the idea that it could represent an evolutionary lineage associated with keratitis (Booton et al., 2009). Moreover, FLA of the genus *Acanthamoeba* can cause severe and chronic infections in humans mainly localized in immune privileged sites, such as the brain and the eye.

Acanthamoeba genotype T4 is known to stimulate proinflammatory cytokines release as well as the early production of IL-10 in innate immune cells, creating an immunosuppressive behavior (Mattana et al., 2016). These facts which might promote the immune evasion of *Acanthamoeba* by inducing a limited inflammatory response *in situ*, together with the accumulating data that indicate that the vast majority of environmental, keratitis, and non-keratitis *Acanthamoeba* isolates

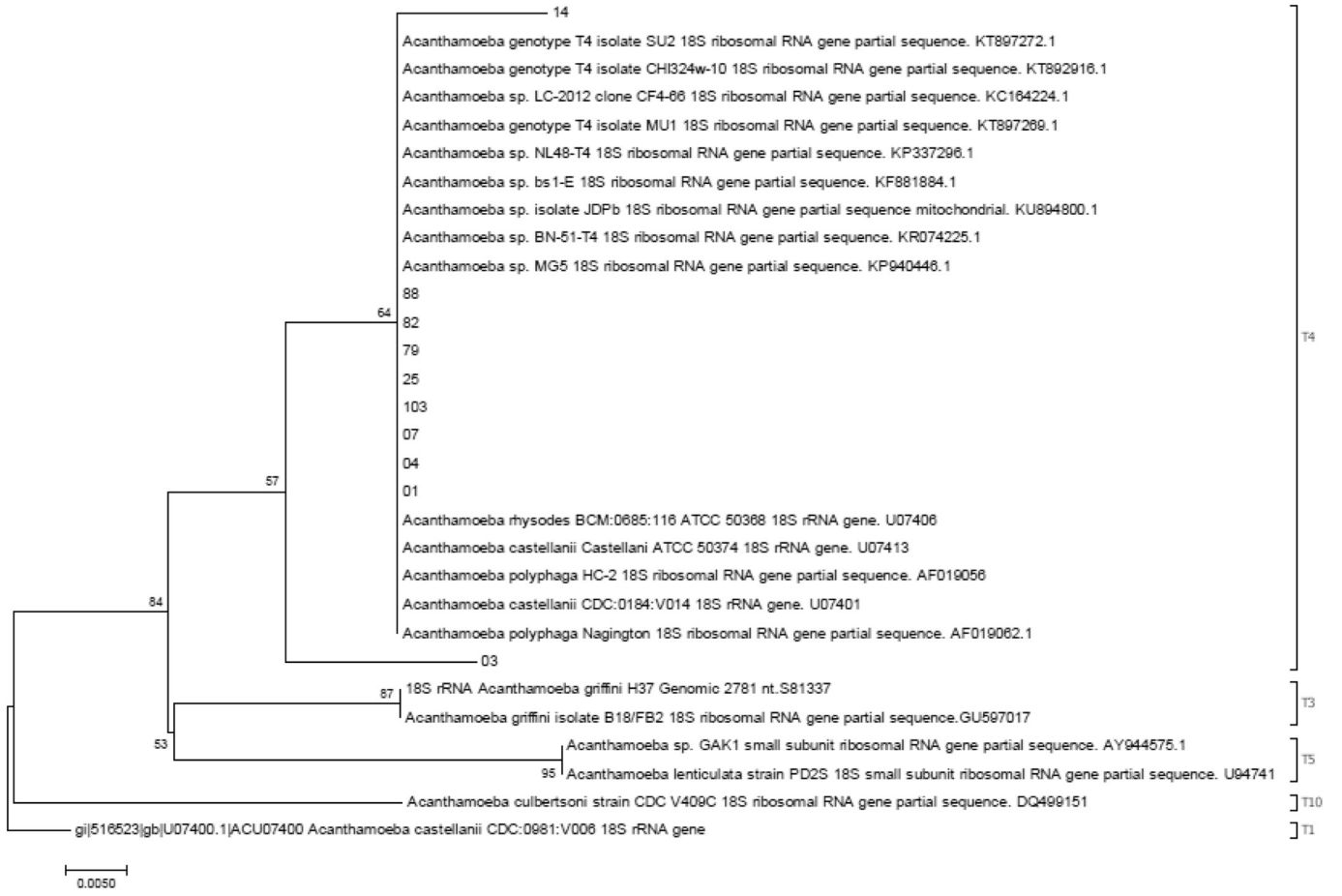

Fig. 5. Phylogenetic tree reconstructed using Kimura two-parameter distance algorithm with 1000 replicates using neighbor-joining method. The sequence of strain *Acanthamoeba* spp. V006, T1 genotype, was used to root the final tree.

belong to genotype T4 (Khan, 2006; Booton et al., 2005; Magliano et al., 2009), certainly reinforces the pathogenic role of this specific genotype. Additionally, full sequence analyses to investigate the phylogenetic relationships of *Acanthamoeba*, revealed *A. polyphaga* as the only species identified in both corneal and CL samples, suggesting the prevalence of this amoeba in our environment. However, in CL wearers the risk for AK acquisition increases substantially because *Acanthamoeba* adhere especially well to the hydrophilic plastic of these lenses and moreover not only *Acanthamoeba* but other FLA, as the one detected in LC 65. These facts, support the AK frequency detected in CL wearers, who most of them stated that they prepared multipurpose homemade cleaning solutions for lens rinsing or did not followed ophthalmologist instructions. Therefore, this explains the relative risk for acquiring AK and the fact that 90% of patients with ocular pathology (ocular abscesses, corneal ulcers, keratitis) who used soft CL, were positive for *Acanthamoeba*, where 65% of our patients referred swimming while wearing CL. These results emphasize the highly preventable nature of CL related AK, thus, improved education for CL wearers and practitioners about hygiene practice and the variable efficacy of contact lens systems could be expected to reduce the incidence of this disease.

The number of reported cases of AK is increasing worldwide every year, due to increasing contact lens use for vision correction and cosmetic purposes. Increased awareness combined with early diagnosis of the disease is currently a good pathway towards better outcomes. However, knowledge about the *Acanthamoeba* pathogenesis and cellular differentiation processes and the development of new nontoxic molecules to human corneal cells are still not fully known and urgently require further investigation in order to provide a better outcome for patients carrying this painful and underdiagnosed infectious disease.

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