EVALUATION OF ACANTHAMOEBA ISOLATES FROM ENVIRONMENTAL SOURCES IN TENERIFE, CANARY ISLANDS, SPAIN

Jacob Lorenzo-Morales¹, Carlos A. Monteverde-Miranda², Concepción Jiménez², María Luisa Tejedor², Basilio Valladares¹, Antonio Ortega-Rivas¹

¹University Institute of Tropical Diseases and Public Health of the Canary Islands, University of La Laguna, La Laguna, Tenerife, Canary Islands, Spain
²Department of Edafology, Faculty of Biology, University of La Laguna, La Laguna, Canary Islands, Spain

Abstract: Tests for potentially pathogenic amoebae were carried out in order to determine the presence of free-living amoebae of genus Acanthamoeba in soil and beach sand sources related to human environments in Tenerife, Canary Islands, Spain. Acanthamoeba identification was based on the morphology of cyst and trophozoite forms and PCR amplification with a genus specific primer pair. The pathogenical potential of Acanthamoeba isolates was characterized by temperature and osmotolerance assays and PCR reactions with two primer pairs related to Acanthamoeba pathogenesis. The results demonstrate the presence of potential pathogenic strain in both sources. Thus, some of the amoebae found in these habitats could act as opportunistic pathogens and may present a risk to human health.

Address for correspondence: Dr. Antonio Ortega Rivas, Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias University of La Laguna, Avda. Astrofísico Fco. Sánchez, S/N, 38203, La Laguna, Canary Islands, Spain.

E-mail: jmlorenz@ull.es

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INTRODUCTION

Acanthamoeba spp. are free-living opportunistic protozoan parasites that pervade the entire environment and can be found in tap, fresh, coastal, and bottled mineral water; contact lens solutions and eyewash stations; soil, dust and air; sewage; heating, ventilation, or air-conditioning units; and gastrointestinal washings [3, 10, 15, 18]. These amoebae are the causative agents of a multifocal encephalitis called Granulomatous Amoebic Encephalitis (GAE), a chronic central nervous system disease of immunocompromised hosts, and various other system disease states including keratitis and pneumonitis. Organisms have been found as members of the normal flora and have been cultured from the upper airways of apparently healthy people, suggesting that infection may be common and self-limited in a competent host [12, 14, 16, 19, 26, 27, 29, 30, 31, 32].

Amoebic keratitis is the only water-related syndrome caused by Acanthamoeba spp. Most episodes of keratitis occur after water exposure or a history of swimming in lakes and ponds while wearing contact lenses and the infection is also linked to non-sterile home-made saline solutions for contact lenses [10, 16, 17, 23, 25].

The identification of Acanthamoeba sp. at the genus level is based on distinctive features of trophozoites and cysts, especially the double walled cyst shape that is unique to the genus. Acanthamoeba species have been classified into distinct morphological groups (I, II, and III) [24]. However, this division of Acanthamoeba sp.
isolates into different species is inconsistent [2, 33]. The taxonomy and classification of the parasite currently are under revision following the successful application of molecular techniques [4, 9, 11, 34, 35].

*Acanthamoeba* pathogenesis is related to several direct and indirect factors. Studies to date have confirmed that binding of *Acanthamoeba* to a host cell is mediated by mannose-binding proteins that leads to secondary events, such as phagocytosis, apoptosis and secretion of extracellular proteases, which play a role causing cellular and tissue damage to the host.

Extracellular proteases, mainly serine and cysteine proteases, are major determinants in the pathogenesis of *Acanthamoeba* infections, being their major role the degradation of host tissues [1, 10].

The aim of this study was to determine the presence of these amoebae soil and beach sand sources in Tenerife, Canary Islands, Spain; and to characterize the pathogenic potential of the isolated strains by biochemical and PCR assays.

### MATERIAL AND METHODS

#### Sample sites and culture of *Acanthamoeba*. Soil samples (ca. 2 g) were collected all over the island of Tenerife and were dissolved in 20 ml of distilled sterile water, and 150 µl of each sample was inoculated onto 2% Neff’s saline non-nutrient agar plates seeded with heat-killed *Escherichia coli*. Sand beach samples were collected during low tide from Tenerife Island beaches. Wet sand from beaches was collected from the top 10 cm of sand located halfway between the high-tide mark and current water level, and dry sand was sampled from the top 10 cm of sand 5 m above the high-tide mark and samples were processed as previously described [5]. In both samples, swabs were stroked on 2% Neff’s saline non-nutrient agar plates within an area 40 x 40 mm in size and incubated at 25°C for up to 2 weeks. These plates were monitored for out-growth of *Acanthamoeba* microscopically and blocks containing *Acanthamoeba* were removed and the amoeba clone by dilution. *Acanthamoeba* isolated in this way were then transferred into axenic cultures by placing the amoebae into PYG medium (0.75% Proteose peptone (wt/vol), 0.75% yeast extract (wt/vol), and 1.5% glucose (wt/vol)).

Amoeba controls from American Type Culture Collection (ATCC) and Culture Collection of Algae and Protozoa (CCAP) (Tab. 1) were grown without shaking in PYG medium at 25ºC.

#### DNA extraction. *Acanthamoeba* trophozoites were harvested at a density of 2 x 10^7 parasites/ml. The cells were pelleted (1,000 g) for 10 min at room temperature and washed 3 times with phosphate-buffered saline (PBS), pH 7.2. Cell pellets were resuspended in lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) and incubated at 55°C for 1 h with 0.25 mg/ml Proteinase K. The DNA samples were purified from amoebae isolates by the phenol-chloroform method [28].

#### PCR amplifications and product analyses. The DNA amplification reactions were performed, using the genus specific, the pathogenic potential related, the serine-protease and the *A. astronyxis*, *A. divionensis* and *A. polyphaga* specific primer pairs [7, 8, 20, 21, 22, 35] in a 30 µl volume containing 10 ng template DNA, 50 mM Buffer KCl; 10 mM Tris-HCl, 2.5 mM MgCl₂, 200 mM dNTP, 0.6 mM each primer, and 0.4 units of *Taq* DNA polymerase (Applied Biosystems, New Jersey), pH 8.3 in a Perkin-Elmer 9600 thermocycler. The cycling conditions were: an initial denaturing phase of 94°C for 1 min and 30 repetitions at 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. The primer extension phase was prolonged for 10 min at 72°C in the last cycle. Amplification products were fractionated by 2% agarose electrophoresis stained with a solution of 0.5 mg/ml of ethidium bromide and visualized under UV light.

#### Osmotolerance and temperature assays. Osmotolerance and temperature assays were developed as previously described [9].

### RESULTS

Amoebae from genus *Acanthamoeba* were identified in 43 of 114 (37.7%) soil samples and in 21 of 50 (42%) sand beach samples by morphology and PCR with the genus specific primer pair (Tab. 2).

To determine pathogenic potential of *Acanthamoeba* isolates, PCR reactions with pathogenic capacity primer pair and serine-protease primer pair were developed. Temperature and osmolarity assays were also developed to distinguish between pathogenic and non-pathogenic isolates [6, 9].

39 of the 43 (90.6%) *Acanthamoeba* strains isolated from soil sources, demonstrated pathogenic potential as
these isolates showed osmotolerance, thermotolerance and pathogenic capacity (Tab. 2). 21 of the 39 (53.8%) Acanthamoeba strains which demonstrated pathogenic potential gave positive amplification with serine-protease primer pair, showing that these strains were pathogenic and virulent (Tab. 2).

Regarding beach sediments samples, 21 of the 21 (100%) isolated Acanthamoeba strains showed pathogenic potential after the osmotolerance, thermotolerance and pathogenic capacity PCR positive results. 21 of the 21 (100%) strains were identified as pathogenic and virulent as they gave positive amplification with serine-protease primers. After PCR reactions with the species specific primer pairs, A. astronyxis and A. polyphaga strains were identified both in soil and beach sources (Tab. 2).

**DISCUSSION**

Acanthamoeba are found in diverse habitats, thus it is not surprising that we often come across and interact with these organisms. Previously, Chappel et al. [6] showed that more than 80% of the normal human population exhibited antibodies against Acanthamoeba. This clearly indicated that these are organisms which often come in contact with humans. Acanthamoeba pathogenicity is a sum of multiple processes which must come together in time and space for the successful transmission of pathogens to a susceptible host, overcome hosts barriers and cause disease. Recent findings in the factors and mechanisms involved in Acanthamoeba pathogenesis may provide tools for the development of epidemiological and therapeutic studies. These amoeba enzymes may have evolved for survival under stress conditions, and nutritional purposes being these conditions important in host infection [1, 2, 6]. Our results confirmed the importance of serine proteases in the pathogenesis of these amoebae as well as the relationship between the adaptation of Acanthamoeba spp. to stress conditions and their capacity to cause disease, as we previously reported in aquatic habitats; in that work, the same species were isolated both in tap and sea water in the same geographical sources [13]. Thus, the potential pathogenicity found in Acanthamoeba sp. strains isolated from soil and beach sources is apparently higher due to the major evolution of the mechanisms that the amoebae have to resist in stress conditions.

In our study, we found a high percentage of potential pathogenic amoebae in both sources; this represents a sanitary risk in these environmental sources. The molecular and biochemical assays demonstrated the presence of these opportunistic pathogens, and the methods that were developed proved to be very useful for the characterization of potential pathogenic Acanthamoeba strains from water samples, or any other environmental sample.

### Table 2. Acanthamoeba isolates found in this study.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>N</th>
<th>Geographic source</th>
<th>Pathogenecity PCR</th>
<th>Protease PCR</th>
<th>Temperature</th>
<th>Osmolarity</th>
<th>Species</th>
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<tbody>
<tr>
<td>SST</td>
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<td>La Orotava</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>SST</td>
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<td>Arona</td>
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<td>+</td>
<td>-</td>
<td>Acanthamoeba sp.</td>
</tr>
<tr>
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<td>Buenavista</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>A. polyphaga</td>
</tr>
<tr>
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<td>3</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>SST</td>
<td>2</td>
<td>Vilallor</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>SST</td>
<td>2</td>
<td>El Tanque</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</tr>
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</tr>
<tr>
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</tr>
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<td>2</td>
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<td>+</td>
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</tr>
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<td>-</td>
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<tr>
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</tr>
<tr>
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<td>Buenavista</td>
<td>+</td>
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<td>+</td>
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</tr>
<tr>
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<td>2</td>
<td>Candelaria</td>
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<td>+</td>
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<td>+</td>
<td>A. astronyxis</td>
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<tr>
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<td>Tacoronte</td>
<td>+</td>
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<td>+</td>
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<td>A. polyphaga</td>
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<tr>
<td>BST</td>
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<td>Santa Cruz</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Acanthamoeba sp.</td>
</tr>
<tr>
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<td>3</td>
<td>Santiago del Teide</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Acanthamoeba sp.</td>
</tr>
</tbody>
</table>

*a Soil Source Tenerife (SST); *b Beach Source Tenerife (BST); *c Growth of Acanthamoeba isolates at 37°C; *d Growth of Acanthamoeba isolates with 1M mannitol.
These findings will serve as additional evidences for the presence of pathogenic Acanthamoeba strains in habitats related to human population, representing a risk for human health.

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