ORIGINAL ARTICLES AAEM

Ann Agric Environ Med 2005, 12, 233–236

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

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Lorenzo-Morales J, Monteverde-Miranda CA, Jiménez C, Tejedor ML, Valladares B, Ortega-Rivas A: Evaluation of *Acanthamoeba* isolates from environmental sources in Tenerife, Canary Islands, Spain. *Ann Agric Environ Med* 2005, **12**, 233–236.

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.

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Key words: pathogenic amoebae, *Acanthamoeba* spp.

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 Amebic 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.

Received: 12 April 2005
Accepted: 12 November 2005

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 ×40 mm in size and incubated at 25ºC for up to 2 weeks. These plates were monitored for out-growth of *Acanthamoeba* microscopically and blocks containing *Acanthamoeba* were removed and the amoebae cloned 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 2×10^7 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 55ºC for 1 h with 0.25 mg/ml Proteinase K. The DNA samples were purified from amoebae isolates by the phenol-chloroform method [28].

Table 1. *Acanthamoeba* strains used in this study.

No.	Species	Strain	Source
1.	*A. astronyxis*	ATCC[a] 30137	Soil (USA)
2.	*A. astronyxis*	CCAP[b] 1534-1	Water (USA)
3.	*A. castellanii*	ATCC 30010	Soil (USA)
4.	*A. castellanii*	ATCC 50492	Keratitis (India)
5.	*A. castellanii*	ATCC 50498	Human cornea (USA)
6.	*A. divionensis*	ATCC 50238	Type strain clone (UK)
7.	*A. divionensis*	ATCC 50251	Type strain clone (UK)
8.	*A. polyphaga*	ATCC 30461	Human cornea (USA)
9.	*A. polyphaga*	ATCC 50495	Human cornea (USA)
10.	*A. polyphaga*	ATCC 30899	Respiratory swab (USA)
11.	*A. polyphaga*	CCAP 1501-18	Soil (USA)
12.	*A. quina*	ATCC 50241	Type strain clone (UK)

[a] American Type Culture Collection; [b] Culture Collection of Algae and Protozoa.

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 $MgCl_2$, 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 94ºC for 1 min and 30 repetitions at 94ºC for 1 min, 55ºC for 1 min, and 72ºC for 1.5 min. The primer extension phase was prolonged for 10 min at 72ºC 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

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

Isolate	N	Geographic source	Pathogenecity PCR	Protease PCR	Temperature[c]	Osmolarity[d]	Species
SST[a]	1	La Orotava	+	+	+	+	*Acanthamoeba* sp.
SST	2	La Orotava	+	+	+	+	*A. polyphaga*
SST	4	Arona	+	-	+	-	*Acanthamoeba* sp.
SST	1	Buenavista	+	-	-	-	*A. polyphaga*
SST	3	Santiago Teide	+	+	+	+	*Acanthamoeba* sp.
SST	2	Vilaflor	+	-	+	-	*Acanthamoeba* sp.
SST	2	El Tanque	+	-	+	-	*Acanthamoeba* sp.
SST	7	La Laguna	+	+	+	+	*A. polyphaga*
SST	2	Los Realejos	+	-	+	-	*A. polyphaga*
SST	2	La Matanza	+	-	+	-	*A. polyphaga*
SST	2	La Victoria	+	-	+	-	*A. polyphaga*
SST	3	Guía Isora	+	-	-	-	*A. polyphaga*
SST	8	Santa Cruz	+	+	+	+	*Acanthamoeba* sp.
SST	2	Chinyero	-	-	-	-	*Acanthamoeba* sp.
SST	2	Puerto Cruz	-	-	-	-	*Acanthamoeba* sp.
BSTb	4	Adeje	+	+	+	+	*Acanthamoeba* sp.
BST	5	Arona	+	+	+	+	*Acanthamoeba* sp.
BST	1	Buenavista	+	+	+	+	*Acanthamoeba* sp.
BST	2	Candelaria	+	+	+	+	*A. astronyxis*
BST	2	Tacoronte	+	+	+	+	*A. polyphaga*
BST	4	Santa Cruz	+	+	+	+	*Acanthamoeba* sp.
BST	3	Santiago del Teide	+	+	+	+	*Acanthamoeba* sp.

[a] Soil Source Tenerife (SST); [b] Beach Source Tenerife (BST); [c] Growth of *Acanthamoeba* isolates at 37ºC; [d] Growth of *Acanthamoeba* isolates with 1M mannitol.

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 amoebae 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 pathogenecity 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.

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.

Acknowledgements

This work was supported by a PhD grant from the Dirección General de Universidades e Investigación del Gobierno de Canarias and by a Redes Temáticas de Investigación Cooperativa Project #C0304 titled Enfermedades Tropicales: De La Genómica Al Control (FIS).

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