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PATHOGENIC FREE-LIVING AMOEBAE OF THE GENERA *ACANTHAMOEBA* IN RECREATIONAL WATER IN THE HUASTECA POTOSINA, MEXICO

AMEBAS PATÓGENAS DE VIDA LIBRE DEL GÉNERO *ACANTHAMOEBA* EN AGUAS RECREATIVAS EN LA HUASTECA POTOSINA, MÉXICO

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ABSTRACT

Studies accomplished in freshwater demonstrate the importance of identify the presence of protozoa like free living amoebae (FLA). In particular, the genera *Acanthamoeba* is associated with severe infections in man, as the Granulomatous Amebic Encephalitis (GAE). The most important factor for the development of these organisms is the high temperature of the water body. The region of the Huasteca Potosina in Mexico, with a tropical climate and great aquatic resorts, like rivers, waterfalls and pools of thermal waters, that allows the development of amoebae. In this study we evaluated the presence of amoebas in the most visited places on the Huasteca Potosina. Samples of a liter were taken in nine sites during the rainy and dry season. 54 strains of amoebas were identify, 46 belong to the genera *Acanthamoeba*, resulting 30 of them pathogenic in the animal tests. The pathogenic isolated amoebas were present in the most attended resorts by the people in the waterfalls or pools of the places sampling. Temperature turned out to be the most important factor for the presence of amoebae.

Key Words: *Acanthamoeba*, free-living amoebae, fresh water isolation, pathogenic.

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INTRODUCTION

The free living amoebae (FLA) are a small group of protozoa of great ecological and medical importance. They are opportunistic microorganisms capable to produce infections in the central nervous system (CNS) and eyes (Martínez, 1993; Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004). The FLA can be found in the whole biosphere including polar caps, soil, water and air, mainly living in aquatic systems like ponds, rivers, streams, lakes, pools, systems of treatment of residual water, underground currents and even fresh water and bottled water (Wellings et al., 1977; Rivera et al., 1981; Rivera et al., 1987a; Rivera et al., 1993; Rivera et al., 1994). To describe the amoebas that can survive in the atmosphere and to be endoparasites the term *amphizoic* it is also used (Page, 1988).

Water temperature between 30 and 40°C enables the proliferation of FLA on natural and recreational thermal waters, as well as industrial discharges thermally contaminated (Kadlec et al., 1980; De Jonckheere, 1981; Rivera et al., 1989a; John, 1993; Visvesvara et al., 2007).

The study of the FLA has demonstrated that a very restricted group only causes infections in humans including the genera *Naegleria, Acanthamoeba, Balamuthia, Sappinia* and probably *Hartmannella* (Rivera and Medina, 1984a; Visvesvara et al., 1993; Martínez and Visvesvara, 1997; Sharma et al., 2000; Marciano-Cabral and Cabral, 2003; Seal, 2003; Kilvington et al., 2004; Khan, 2006).

Some of the diseases dues of the genera *Acanthamoeba*, are granulomatous amebic encephalitis (GAE), keratitis (AK), otitis, uveitis, cutaneous and nasopharyngeal infections. *Acanthamoeba* keratitis is a painful, potentially blinding corneal infection which is difficult to treat, frequently misdiagnosed and commonly associated with contact lens wear (Marciano-Cabral and Cabral, 2003; Kilvington et al., 2004; Schuster and Visvesvara, 2004; Visvesvara et al., 2007). In particular, Granulomatous Amebic Encephalitis (GAE) is caused by infections with: *Acanthamoeba polyphaga, Acanthamoeba castellanii, Acanthamoeba palestinensis, Acanthamoeba rhysodes, Acanthamoeba astronyxis, Acanthamoeba culbertsoni* and *Acanthamoeba hatchetti*.

The etiologies agents of *Acanthamoeba* keratitis, until described today are *A. castellanii, A. culbertsoni, A. polyphaga, A. hatchetti* and *A. rhysodes* (Bonilla et al., 2000).

Although the true incidence of human infection with these amoebae is not known, over 112 worldwide cases of GAE have been reported (Marciano-Cabral and Cabral, 2003; Kilvington et al., 2004).

In Mexico, 29 cases have been reported recently (Bonilla and Ramírez, 2008; Bonilla and Ramírez, 2011). Only five of them are documented, four of them were contact lens wearers (three soft, one hard lens); one patient admitted to wearing their lenses while swimming (Omaña et al., 2005).

In Mexico, natural resorts are preferred in vacations. The Huasteca Potosiona zone has many places of this kind for tourists, however they lack of control checkpoints of microorganisms at water bodies, like chloride treatment and maintenance. Protozoa infections by FLA are an important health issue considering the lack of efficient treatments and its wide distribution. The increasing number of clinical cases suggests new strategies for identification and opportune diagnosis of FLA infections.

MATERIAL AND METHODS

Sampling site

Experimental samples were collected in sterilized flasks, from the nine resorts with natural or artificial water body in the Huasteca Potosina, two samplings in the rainy months and two samplings in the dry season (Table 1), in the most attractive places for visitors. Samples were lately analyzed in laboratory.

Physicochemical parameters determined *in situ*

Dissolved Oxygen (DO), temperature, chloride, using Mini Analyst Chlorine, free and total, Mod. 942-001, Orbeco-Hellige.

The obtained data were compared with the optimum values average for the natural waters in operational conditions reported previously (APHA, 1998).

Isolation and culture of FLA

The samples were filtered through 8µm Millipore membranes and inoculated in plates with agar not-nutritive by duplicate, previously covered with a layer of *Escherichia coli* inactivated to 65-70ºC, during 30 minutes (NNE agar). Plates were incubated at 37 or 42ºC, to obtain the best grown of strains, and then were maintained in a culture stock in fresh NNE agar plates by 24 hours (Bonilla et al., 2000).

Identification of the strains

Strains were cultured in axenic medium Bactocasitona and PBSCG enriched with 10% fetal bovine serum (Rivera et al., 1984b; Rivera et al., 1987a), for triplicate at 37ºC. The identification of FLA was made by observation to the microscope of internal structures of trophic and cysts forms, and the use of a taxonomic key (Page, 1988).

Animal pathogenicity test

A 0.02-ml aliquot of axenic medium containing from 10^4 to 10^6 amoebae was inoculated in three-week old CD-1 mice, according to Rivera et al. (1994), by intranasal (IN) and intracerebral (IC) via. Signs of GAE were observed after 21 days characterized by mobility, breathing, sweating, reflections and water intakeand, mice were killed by carbon dioxide chamber (Cerva et al., 1973). Portions of the brain, liver, lungs and kidneys were inoculated onto NNE plates to retrieve the strains. A pathogenic strain is considered when 50% of infected animals died before 21 days.

Statistical methods

Pearson´s correlation coefficient was applied to look for correlation between *Acanthamoeba* isolates and physicochemical variables.

RESULTS

Physicochemical parameters determined *in situ*

In the rainy season (Table 2), the values of environmental temperature ranged from 24º to 30ºC. The water temperature was from 23º to 37ºC, and pH varied from 6.4 to 8.1. The highest levels of oxygen (DO) were obtained in the natural sites samplings (6.8 to 7.8 mg/L), and the lowest was in Taninul hotel (0.5 mg/L). In the swimming pool the free residual chlorine was under detection limits (Table 2). In the dry season (Table 3) the environmental temperature ranged from 24º to 31º C. The water temperature was from 19º to 36ºC, and pH varied from 6.4 to 7.7. The levels of oxygen (DO) were very high in two waterfalls (8.0 to 8.6 mg/L), and Taninul hotel was the lowest level (0.5 mg/L). In the swimming pool the free residual chlorine was under limit detection in both seasons.

Identification of FLA strains

49 strains of *Acanthamoeba* genera were isolated from the nine water bodies in both seasons, either raining or dry season (Table 1). 17 strains corresponded to *Acanthamoeba polyphaga*, 21 to *Acanthamoeba castellanii*, two to *Acanthamoeba astronyxis*, one *Acanthamoeba rhysodes* and eight were not determined.

Animal pathogenicity test

From the 49 amoebas strains isolated, 30 from genera *Acanthamoeba* showed positive pathogenicity in both infection methods, and 16 resulted negatives. Three strains could not be determined due to insufficient grown.

Statistical methods

A Pearson´s correlation coefficient showed that there was a significant correlation (p<0.05) between physicochemical parameters *A. polyphaga* and *A. castellanii*. A significant correlation between *A. castellanii* (p= 0.474), *A. polyphaga* (p= 0.726) and water temperature was found.

Table 1. Isolates from water bodies on the Huasteca Potosina: morphological identification and pathogenicity tests on mice. Cascaditas and Xilitla places did not present.

Sampling sites	Strains isolated	Optimum growth temperature (ºC)	Morphological diagnosis	Pathogenicity tests IC IN
Media Luna Lagoon	3	37	*A. polyphaga*	3 / IC (+) IN (-)
	3		*A. castellanii*	3 / IC (+) IN (-)
	1		*Acanthamoeba sp.*	1 / IC (-) IN (-)
Cascaditas river	0	nm	-------------	-------------------------
Tamasopo waterfall	2	37	*A. polyphaga*	2 / IC (+) IN (+)
	3		*A. castellanii*	1 / IC (+) IN (+)
Taninul Hotel pool	3	37	*A. polyphaga*	2 / IC (+) IN (+)
	3		*A. castellanii*	2 / IC (+) IN (+)
	1		*Acanthamoeba* sp.	1 / IC (-) IN (-)
	1		*A. astronyxis*	1 / IC (-) IN (-)
Xilitla	0	nm	-------------	-------------------------
Tankanhuitz Pool	1	37	*A. polyphaga*	1 / IC (+) IN (+)
	1		*A. castellanii*	2 / IC (+) IN (+)
	1		*Acanthamoeba sp.*	1 / IC (-) IN (-)
Tambaque borne river		37		
	1		*A. polyphaga*	1 / IC (+) IN (+)
	1		*A. castellanii*	1 / IC (+) IN (+)
	1		*Acanthamoeba sp.*	1 / IC (-) IN (-)
	1		*A. astronyxis*	1 / IC (-) IN (-)
Swimming pools "El Bañito"		37		
	6		*A. polyphaga*	5 / IC (+) IN (+)
	4		*A. castellanii*	4 / IC (+) IN (+); 1(-)
	2		*Acanthamoeba sp.*	2 / IC (-) IN (-)
	1		*A. polyphaga*	1 / IC (+) IN (+)
Swimming pools "El Gogorron"		37		
	6		*A. castellanii*	4 / IC (+) IN (+)
	2		*Acanthamoeba sp.*	2 / IC (-) IN (-)
	1		*A. rhysodes*	1 / IC (-) IN (-)

nm: not measured.
IC: Intracerebrally
IN: Intranasally.

Table 2. Values of physical-chemical parameters in water bodies of Huasteca Potosina in rainy season. Data are expressed as mean ± SD of two samples.

Sampling sites	pH	Air temperature (ºC)	Water temperature (ºC)	Dissolved oxygen (mg/L)	Free residual Cl (mg/L)
Media Luna	6.6	30	26	1.4	0
Cascaditas	7.6	29	26	7.8	0
Tamasopo	8.1	28	28	7.2	0
Taninul Hotel	6.7	30	35	0.5	udl
Xilitla	7.8	24	23	7.5	0
Tankanhuitz	7.5	29	25	6.8	0
Tambaque	7.4	30	26	7.6	0
"El Bañito"	7.1	30	33	1.2	udl
"El Gogorron"	7.7	30	37	3.5	udl

udl: under detection limits.

Table 3. Values of physical-chemical parameters in water bodies of Huasteca Potosina in dry season. Data are expressed as mean ± SD of 2 samples.

Sampling Sites	pH	Air temperature (ºC)	Water temperature (ºC)	Dissolved oxygen (mg/L)	Free residual Cl (mg/L)
Media Luna	6.4	30	28	1.8	0
Cascaditas	7.2	24	22	8.0	0
Tamasopo	7.4	26	24	8.6	0
Taninul Hotel	6.8	30	35	0.5	0
Xilitla	7.3	22	19	7.4	0
Tankanhuitz	7.5	27	25	4.5	0
Tambaque	7.3	28	26	7.9	0
"El Bañito"	7.2	27	30	1.7	udl
"El Gogorron"	7.7	31	36	3.9	udl

udl: under detection limits.

DISCUSSION

Since the first medical reports of infection and morbidity by FLA, increasing evidences supports the importance of water analysis as a strategy to prevent and detect possible infections (De Jonckheere et al., 1991). This fact has greater importance considering that the FLA potentially pathogens are found with facility in water bodies of industrial and recreational use (Coronado-Gutiérrez and López-Ochoterena, 1980; Rivera et al., 1989a).

In the present report was observed the presence of four different species of *Acanthamoeba* more frequently in closed water deposits than natural open resorts (Table 1). At swimming pools "El Bañito" was detected over 10 strains of *Acanthamoeba* with a high pathogenicity, indicating a serious health hazard for the local population and visitors. This could be a consequence of flow currents throw the water body, the chemical composition, as well as the numbers of visitors in each place. It is interesting that several detections presented a positive result to the intranasal pathogenicity tests, suggesting a potential risk of infection for further tourists. The isolation of pathogenic strains of *Acanthamoeba* from the water bodies of the Huasteca Potosina presents an important evidence of the potential exposure to amoebas, increasing the risk to develop GAE and other diseases in local population. There are not evidences of GAE infections through nasal via, except thermophilic amoeba *Naegleria fowleri*, pathogenic agent of primary amoebic Meningoencephalitis (PAM) that is more frequent during the hottest summer months in temperate countries. *Acanthamoeba* are opportunistic pathogen than can remain in body without effects, until occurs another infection or immune-compromised that allow the GAE (Martínez, 1993; Visvesvara et al., 2007).

The temperature values of the water during the samplings denote a considerable difference between the rainy and dry season (Tables 2 and 3). The natural water resorts on the Huasteca present higher temperatures that explain the presence of this kind of amoebas, independently of the weather or season. Pathogenic amoebae of the *Acanthamoeba* genera are more common in water bodies with high temperatures (over 37°C) that favor its proliferation, as mention Rivera et al. (1989a), Visvesvara and Stehr-Green (1990), John (1993) and Bonilla et al. (2000), because *Acanthamoeba* grows better in these conditions, due, *Acanthamoeba* have the capacity to form cysts that are resistant to dehydration, cold and chlorination, due to the fact that the wall of the cyst contains cellulose (Page, 1988). Most of the environmental isolations have been made from habitats that have been polluted by humans or from thermal effluents, hot springs and waters with natural or artificial high temperatures. Few microorganisms can resist such temperature, allowing that thermophilic amoebae can proliferate almost without competition. *Acanthamoeba* has the capacity to form cysts resistant to dehydration, low temperatures and chlorination (Page, 1988). It is possible to find it in anybody water for recreational uses in this latent stage and represents a potential risk to health (Bonilla and Ramirez, 2011). In future, it would be interesting to correlate the number and characteristics of users at these places, with the temperature change, water oxygenation and the presence of *Acanthamoeba*.

In the Huasteca Potosina hot springs and swimming pools with thermal water bodies are numerous and although many people may be exposed to GAE. Though they are few reported cases. The factors that determine the virulence and resistance of GAE are as yet unclear, as well as the diseases that they cause. The total elimination of *Acanthamoeba* of the aquatic systems is not feasible, since is needed to develop integral plans to control its development. The high levels of DO and pH of water bodies does not represent limiting factors for the growth of *Acanthamoeba* (Bonilla and Ramirez, 2011). The low-flow currents in the water bodies and the reduced levels of oxygenation, beside the lack of bacteriological control and maintenance could enhance the proliferation of microorganisms in the recreational places, even after treatments with chemicals in any season (Tables 2 and 3).

We can concluded that human manipulation of water resources increase probability of infection by *Acanthamoeba* in recreational temperate swimming pools, where chlorination is inadequate. The tropical and subtropical conditions of the nature water bodies from the Huasteca Potosina allow the proliferation of amoebas as *Acanthamoeba*, and that would constitute a human health hazard, similar to PAM infection produced by *Naegleria*.

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