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Full Length Research Paper

Isolation and identification of pathogenic *Acanthamoeba* using morphological method combined with PCR, in recreational water bodies in the State of San Luis Potosí, Mexico

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The free-living amoebae (FLA) can be found in any type of environment, but specifically the genus *Acanthamoeba* is associated with several infections as granulomatous amoebic encephalitis and keratitis amoebic, typical related to the use of contact lenses. To determinate the presences of FLA in a specific media or in infected individuals require at least one week identifying them. In this investigation we used the traditional method of taxonomic identification, combined with the PCR technique to determinate the presence of pathogenic FLA of the genus *Acanthamoeba* in aquatic environments located in the Huasteca Potosina. Four independent samplings in nine sites were accomplished throughout an annual cycle in The Huasteca zone of the State of San Luis Potosi, including springs, waterfalls, lagoons, and pools fed with thermal waters. 74 collected samples, and, by duplicate were processed by the conventional laboratory methods to detect organizations belonging to the genus *Acanthamoeba* and analyzed by the technique of PCR, using two primers, one for the detection of *Acanthamoeba* genus, and the second for the detection of pathogenic strain. In seven selected places, 49 isolations of the genus *Acanthamoeba* were obtained by the traditional method, most of them were from the recreational center ponds fed with thermal water. The study of pathogenicity in mice showed 31 cases. PCR analysis with primer Ac6 revealed 33 pathogenic samples, the same result obtained by the conventional method. The use of primer GP (P2) confirmed 41 samples with the presence of *Acanthamoeba* genus previously identified, demonstrating that PCR technique is a rapid and reliable alternative to detect FLA. Their presence in popular resorts and touristic natural water bodies is an alert of a potential health care risk.

Key words: Free-living Acanthamoeba, morphological identification, PCR, recreational water bodies.

INTRODUCTION

The free-living Amoebae (FLA) are found in all type of environment due to its importance in the food chain within natural communities on water, soil, even in the air (Rivera et al., 1987; Bonilla-Lemus et al., 2014). Originally this group of protozoa was denominated "limax" (viscous form), conformed by soil amoebas, free-life and "amphizoics" amoebas (Page 1977), and strongly defined as parasite of the central nervous system (CNS), corneal

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epithelial (Kilvington et al., 1990; Moon et al. 2008), vagina and prostate in the human and some animals (Martínez 1993). In specific, a group of FLA is the cause of such infections, including amoebas of genera *Naegleria, Acanthamoeba* and *Balamuthia* (Visvesvara et al., 1993; Méndez et al., 1996), *Sappiniadiploidea* (Górnik and Kuźna-Grygiel, 2004), last-mentioned specie recognized after wards by (Qvarnstrom et al., 2009) as *S. pedata* (Qvarnstrom et al., 2009) and probably *Hartmannella* (Centeno et al., 1996; Kuiper et al., 2006).

The increasing concern over pathogenic FLA is due to its association with severe and deathly pathologies in humans (Martínez and Visvesvara, 1997) including disorders at CNS like primary amoebic meningoencephalitis (PAM) or naegleriosis, and the granulomatous amoebic encephalitis (GAE) or acanthamoebosis. The cause, etiology agent, pathogenicity, damage sites, course and clinic demonstrations of these diseases are different (John, 1993), usually associated to AIDS patients or with immune-compromised individuals (Marciano-Cabral and Cabral, 2003). Over 150 cases of GAE caused by *Acanthamoeba* have been published worldwide, but less than 10 patients have survived (Schuster and Visvesvara, 2004), and the number of keratitis amoebic (KA) cases by the same parasite have increased dramatically in recent years (Visvesvara, 2003). These reports indicate a potential health problem and support the importance of the initial differential diagnoses to provide an accurate treatment before the fatal consequences (Martínez, 1993).

Among the lack of knowledge about PAM and GAE, the conventional methods for the identification of FLA are time consuming and require a professional training to get an accurate and reliable result. The aim of this work is to develop a fast and precise identification method of pathogenic FLA, based on modern PCR technique, in recreational water bodies of the touristic zone named The Huatesca in Mexico.

MATERIALS AND METHODS

Sample collection

Nine recreational places in The Huasteca, San Luis Potosi, were selected according to the local populace and visitors. Many of them receive only one income flux of water, geothermic warmed, and usually static. Seventy-four samples with duplicate were collected in 1 L sterile propylene bottles from the surface of the water body (<10cm below), and preserved at ambient temperature before process in the laboratory.

Acanthamoeba detection

As pretreatment, samples were filtered in a 8 µm Millipore mesh, and the membranes were put on Petri dish containing NNE culture media (De Jonckheere, 1977; Page, 1988), maintained invested and in plastic bags. For duplicates, the membranes were washed on a sterile tube with 5ml of SEP solution (sucrose-EDTA) and suspensions were frozen at-20°C until DNA extraction. All experiments were carried out under sterile condition.

Isolation and culturing of *Acanthamoeba*

The membranes were withdrawn from the agar plaque and observed in microscope, locating the amebic growth zones. These colonies were transferred to NNE media by duplicate and incubated at room temperature (20-22°C) and 37°C during 24 h. From each plate amoebas were isolated form onoaxenic culture in the same conditions, and then transferred to PBSGM media (modified by Rivera et al., 1987) and Bactocasitona media (BC)supplemented with 2 or 10% of fetal bovine serum, incubated at 30°C (Page, 1988; Martínez, 1985).

Morphologicaldiagnosis

The morphologic identification of *Acanthamoeba* was carried out accomplishing live preparations from the axenic culture. *Acanthamoeba* isolates were identified on the basis of the morphological features of trophozoites and cysts, growth temperature and pathogenicity and using the taxonomic key of Pussard and Pons (1977) and Page (1988).

Pathogenicity test

Male *Musmusculus* CD-1mice of three weeks-old were obtained from the local animal facility. Animals were maintained in controlled environmental conditions and light-dark cycles (12:12 h), and all experiments were conducted under local animal care and ethical use protocols, in accordance with the Federal Guidelines for the Care and Use of Animals (NOM-062-ZOO-1999 Ministry of Agriculture, Mexico) and approved by the Institutional Ethics Committee of the National Autonomous University of Mexico's Faculty of Higher Studies Iztacala.

Five mice were inoculated with the trophozoites from the axenic culture by route interbrain or nasal installation, in a calculated account of 1×10^3 - 1×10^6 cells/ml. The inoculated volume was of 20 µl, for the application interbrain was inoculated through the inter parental articulation, in line with ears (Červa et al., 1973; De Jonckheere, 1980). For the nasal installation; trophozoites were applied in the same dose through the nasal orifice. Animals were observed daily during 21 days, registering the changes in behavior. The amebic strains were considered pathogenic when at the end of the test, the mortality in the group of mice was 60% or greater. The surviving animals were sacrificed to extract the brain, and then incubated in the NNE media at 30°C

Table 1. PCR primers design for GP and Ac6 identification

Template	Sequence (5'-3')	Identification
GP(P_2) for.	**$_{1383}$TCCCCTAGCAGCTTGTG$_{1400}$**	Genus
GP(P_2) rev.	**$_{1655}$GTTAAGGTCTCGTTCGTTA$_{1673}$**	
Ac6/10. for.	**GGCGAAGAACCTGCATCAGC CAACCAACTCCC**	Pathogen
Ac6/210. rev.	**GAGCCA**	

for 24-48 h, in order to determine the presence of amebic development. Samples of liver, lungs and kidneys were analyzed with the same protocol, including those dead mice with amebic pathogenic characteristics.

PCR conditions for *Acanthamoeba* and analysis

The extraction of the DNA was accomplished by the method of phenol-chloroform (Molecular Microbial Ecology Manual, 1995). For amplification, we designed the primer GP (P2) of 272-bp, according to the genomic sequence reported by Vodkin et al., (1992) and Lehmann et al. (1998), to identify amoebas of the genus *Acanthamoeba*, and the primer Ac6 of 195-bp, for pathogenic strain, proposed by Howe et al. (1997). PCR was performance in athermo cycler PerkinElmer (GeneAmp PCR System 2400) with a GeneAmp PCR reagent kit (PerkinElmer).

As non-pathogenic control, we included samples from reference strains culture of *A. castellanii* (Neff) and *A. lenticulata* (PD2); and as positive pathogenic control *A. castellanii* (AC), *A.culbertsoni* (LillyA-1), *A. polyphaga* (PQ) and *A. lugdunensis* (SH-565). All pathogenic strain came from the American Type Culture Collection (ATCC).

For the amplification were added 27.5 µl of distilled water, 5 µl of buffer PCR, 8 µl of nucleotides mix (dNTP), 0.5 µl of AmpTaq, 3 µl of $MgCl_2$, 0.5 µl of primer, 5 µl of DNA (template) from the sample or reference strains, giving a total volume of reaction of 50 µl.

Time and temperatures established in the thermo cycler for each cycle were set: 5 min at 95°C, 90 s at 94°C,90 s to 56°C for annealing of primer GP(P2), or 60sec at 64°C for primer Ac6, 90 s at 72°C, 30 total cycles and finally 1 cycle of 5 min at 72°C.

After this, products were analyzed in a 2% agarose gel, using two molecular-weight marker of 100 bp and the second marker ϕX174 RF DNA/*Hae*III.

Both primers are reported by The European Microbiology Laboratory, Gene Bank and the Sequence database Nucleotides (DDBJ) with key of access number M65180 from *Acanthamoeba*, analyzed by homologous in the mentioned data base using the program FASTA of the package GCG; using screens of repetitious DNA (Vodkin et al., 1992). The construction of the primers used in the study is shown in Table 1.

Statistical methods

Pearson´s correlation coefficient was applied to analyze total *Acanthamoeba* isolates with physicochemical variables in all water bodies.

RESULTS

Isolation and morphological diagnosis

From the seventy-four samples processed by the traditional method, 49 genus *Acanthamoeba* isolated were obtained either in the raining or dry season, in seven of the nine places selected. 17 strains corresponded to *A.polyphaga*, 21 to *A. castellanii*, 2 to *A. astronyxis*, one to *A. rhysodes* and 8 were not determined. These amoebas came from the site named the Bañito, 24.4%, from the Gogorrón 20.4 and 16.3% from the resort place Taninul (Table 2).

Pathogenicity test

From the 49 amoebas strains isolated, 25 (51.0%) of them identified as genera *Acanthamoeba* showed positive pathogenicity in both inoculation methods; eleven samples (22.4%) were negative and only six (12.4%) presented a positive pathogenicity after interbrain installation. Seven strains could not be determined due to insufficient growth.

According to pathogenic tests, 31 (63.4%) showed a pathogenic response in mouse.

PCR test for *Acanthamoeba* and sequence analysis.

Identification of amoebas from genus *Acanthamoeba* by DNA extraction and PCR revealed a presence of 83.6% in the different places of isolation (Figure 1), and with the primer Ac6, 67.3% of amoebas coincided with the pathogenicity tests (Figure 2).

A Pearson´s coefficient demonstrated a significant correlation (p<0.05) between physicochemical parameters, *A. polyphaga* and *A. castellanii*. Only a significant negative correlation (-0.24) between amoebae and temperature was found.

Table 2. FLA isolated from water bodies on the Huasteca Potosina: morphological identification and pathogenicity tests on mice. Cascaditas and Xilitla places did not present FLA.

Sampling Sites	AVL Strains isolated	Optimum Growth Temp. (ºC)	Morphological Diagnosis	PCR Ac6/GP(P_2)		Pathogenicity Tests	
						IC	IN
Media Luna	3		*A.polyphaga*	3+	3+	3/IC (+)	IN (-)
Lagoon	3	37	*A.castellanii*	3+	3+	3/IC (+)	IN (-)
	1		*Acanthamoebasp.*	1-	1-	1/IC (-)	IN(-)
Cascaditas	0	Nm	------------			--------------------------	
Tamasopo	2		*A.polyphaga*	2+	2+	2/IC (+)	IN (+)
Waterfall	3	37	*A.castellanii*	1+	1+	1/IC (+)	IN (+)
	3		*A.polyphaga*	2+	2+	2/IC (+)	IN (+)
	3		*A.castellaniiAcanth*	2+	2+	2/IC (+)	IN (+)
TaninulHotel	1	37	*amoebasp.*	1-	1+	1/IC (-)	IN (-)
	1		*A.astronyxis*	1-	1+	1/IC (-)	IN (-)
Xilitla	0	Nm	-------------			--------------------------	
Tankanhuitz	1		*A.polyphaga*	1+	1+	1/IC (+)	IN (+)
Pool	1	37	*A.castellanii*	1+	1+	1/IC (+)	IN (+)
	1		*Acanthamoebasp.*	1-	1-	1/IC (-)	IN (-)
	1		*A.polyphaga*	1+	1+	1/IC (+)	IN (+)
Tambaqueborne	1		*A.castellanii*	1+	1+	1/IC (+)	IN (+)
river	1	37	*Acanthamoebasp.*	1-	1-	1/IC (-)	IN (-)
	1		*A.astronyxis*	1-	1+	1/IC (-)	IN (-)
Swimmingpools	6		*A.polyphaga*	5+	6+	5/IC (+)	IN (+)
"ElBañito"	4	37	*A.castellanii*	4+	4+	4/IC (+)	IN (+)
	2		*Acanthamoebasp.*	2-	2+	2/IC (-)	IN (-)
	1		*A.polyphaga*	1+	1+	1/IC (+)	IN (+)
Swimmingpools	6		*A.castellaniiAcanth*	6+	6+	4/IC (+)	IN (+)
"ElGogorron"	2	37	*amoebasp.*	2-	1+	2/IC (-)	IN (-)
	1		*A.rhysodes*	1-	1+	1/IC (-)	IN (-)

nm: not measured. IC: intracerebrally. IN: intranasally.

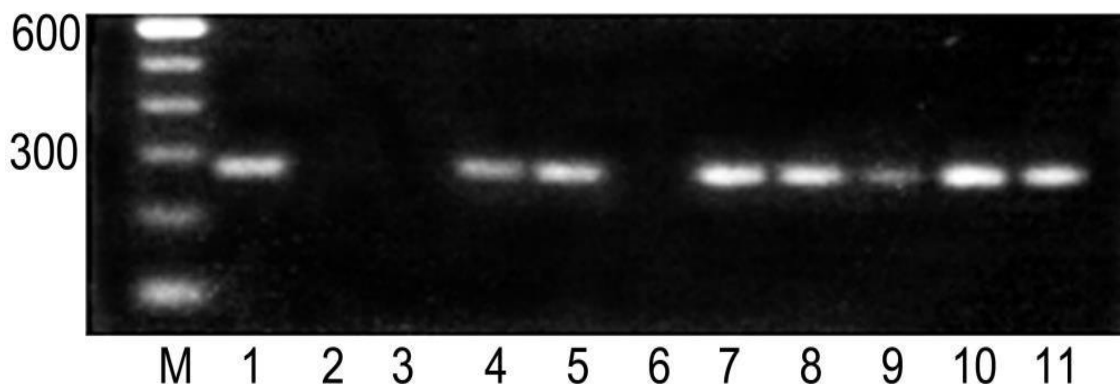

Figure 1. DNA amplification by PCR using primer GP(P_2), of reference strains and water samples from Bañito and Gogorrón. From left to right: molecular weight marker 100bp (M), □X174RF DNA/*Hae*III; 1 *A.castellanii* (AC), 2 negative control, 3 *A. lugdunensis* (SH-565) ,4,5 *A. culbertsoni* (LillyA-1) and *A. polyphaga* (PQ) positive control, 6 *A.castellanii* (Neff) negative control, 7,8,9 samples from Bañito (Ba 1(1), Ba 2(1), Ba 3(1)) and 10,11 samples from Gogorrón (Go1(1) and Go 2(1)). Gel representative of duplicatetest.

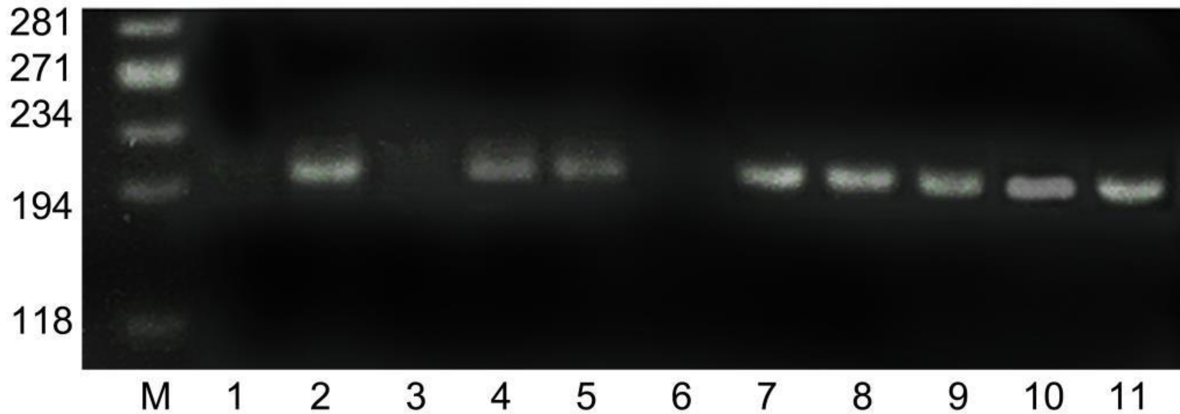

Figure 2. DNA amplification by PCR using primer Ac6, of reference strains and water samples from Bañito and Gogorrón. Molecular weight marker 100 bp (M), X174RF DNA/*Hae*III; 1 negative control; 2 *A.castellanii* (ACpositive control; 3 *A. lenticulata* (PD2) negativecontrol; 4,5 *A. culbertsoni* (LillyA-1) and *A. polyphaga* (PQ) positive control, 6 *A. castellanii* (Neff) negative control, 7,8,9 samples from Bañito (Ba1(1), Ba2(1), Ba3(1)) and10,11 samples from Gogorrón (Go1(1) and Go2(1)). Gel representative of duplicate test.

DISCUSSION AND CONCLUSIONS

FLAs are common in habitants in soil and water bodies (Rivera et al., 1987) usually adhered to floating particles in the surface or sediments (Kyle and Noblet 1986). In this work, the highest number of isolation of *A. canthamoeba* was obtained on three recreational systems with a continuous flow fed directly from water springs. Temporarily the water body is confined in ponds with structures as walls and corners that permit stemmed water and the accumulation of waste. These conditions favor the presence of microhabitats, including FLA grown in a relatively protected area, with sufficient food, bacteria, oxygen concentration, organic matter and adequate temperature. Furthermore, these water bodies are not treated with any additional disinfectant (Bonilla et al., 2000).

According to pathogenicity tests, 63.4% of isolated amoebas showed a positive result in mouse, indicating a potential health risk for visitors of these places (Martínez and Visvesvara, 1997) . Human factor is also important to increase the presence of amoebas to the water, considering the fact that the three pools are surrounded by grass and land, and the continuous transit of bathers increase bacteria and FLA proliferation.

The presences of pathogenic FLA in recreational water bodies increase the probability of hazardous infections. There are reports of GAE in hosts not-immuno compromised, after infections by respiratory tract via or in skinlesions, invading the CNS the sanguine route with a fatal consequence (John 1993; Martínez and Visvesvara 1997). On the other hand, immune competent host scan present *Acanthamoeba* infection without the development of GAE [1]. KA is another infection caused by FLA with an increased frequency since the last decade. In 1994 about 500 cases were registered and in 1998 over 1000 (Martínez and Visvesvara 1997; John 1993; Visvesvara and Stehr-Green 1990; Lares-Villa 2001). KA have been associated with corneal traumatism and to the use of contact lenses, but at present is known as a risk factor of lens contact contamination with water or dust (Martínez and Visvesvara 1997). For these clinical reasons, is important for the development of accurate and reliable methods of identification of FLA from diverse samples.

Few reports have used specific DNA primers for the identification of the genera *Acanthamoeba*, from clinic samples of reference strains culture (Vodkin et al., 1992; Lehmann et al., 1998; Howe et al., 1997), or even less from environmental samples. For the first time, in this work, extraction of the total DNA from recreational water bodies in order to compare and evaluate its usefulness in the identification of these amoebas by PCR technique was applied. The traditional method was result effective for the identification of *Acanthamoeba* only at genera level, but it is a slow process accomplished in 30 days that requires a professional training and experience, delaying the time for an accurate diagnosis (Qvarnstrom et al., 2006).

The implementation of PCR technique has demonstrated its utility for the detection of *Acanthamoeba* in human samples with suspicion of infection. Even with a basic infrastructure, it is possible to implement the same procedure for the detection of other pathogenic FLA as *Naegleria* and *Balamuthia*, in critical occasions when the life or recovery of patients depends on an opportune diagnostic. Unfortunately, most of the cases of *Acanthamoeba* have been diagnosed *postmortem* (Marciano-Cabral and Cabral 2003). The PCR identification could be considered as an expensive technique, nevertheless in the long term is a faster, simpler and more adequate method for the identification of amoebas of the genus *Acanthamoeba* presence in the

environment.

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