The Microscopic Detection and Identification of Acanthamoeba Spp. In Water Samples Following Enrichment by in Vitro-Cultivation Under Two Distinct Temperature Conditions.

Sakhi Elmahdi (Sakhi.elmahdi@gmail.com)
Zoology laboratory university Mohammed V https://orcid.org/0000-0002-0704-1572
MOUMNI MOSTAFA
Institut National d'Hygiene du Maroc
Radid Horia
Mohammed V University of Rabat: Universite Mohammed V de Rabat
ARAHOU MOHAMED
Université Mohammed V de Rabat Institut Scientifique: Universite Mohammed V de Rabat Institut Scientifique
FEKHAOUI MOHAMED
Université Mohammed V de Rabat Institut Scientifique: Universite Mohammed V de Rabat Institut Scientifique

Research Article

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The microscopic detection and identification of *Acanthamoeba* spp. in water samples following enrichment by *in vitro*-cultivation under two distinct temperature conditions.

SAKHI EL MAHDI.[(1)*]*,MOUMNI MOSTAFA.*[3]*,RADID HORIA.*[2]*, ARAHOU MOHAMED.*[1]*, FEKHAOUI MOHAMED.*[1]

1 University Mohammed V, Department of Scientific Institute, Zoology Laboratory, P.O. Box 1014, Rabat, Morocco.

2 University Mohammed V, Department Biology,- 4 Ibn Battouta Avenue, P.O. Box 1014, Rabat, Morocco.

3 National Institute of Hygiene, Department of General Parasitology, P.O. Box 1014, Rabat, Morocco.

Corresponding Author: Department of Scientific Institute, zoology laboratory, Mohammed V University – 4, Ibn Battouta Avenue, P.O. Box 1014, Rabat, Morocco —Telephone number: 00212622494795 e-mail: sakhi.elmahdi@gmail.com

Abstract

Acanthamoeba is a genus of free-living amoeba commonly found in environmental sources such as water, soil and air, and can infect humans. There is a significant challenge in the detection and identification of members of this genus in water samples. In order attempt to overcome this challenge, we investigated using *in vitro* culture, under distinct temperature conditions, to grow and enrich amoeba prior to detection and identification. Aliquots of 150 water samples, collected from Rabat (30 from each river, fountain, sea, public bath and tap water), were individually inoculated into standard culture medium non-nutritive agar and incubated for two weeks at 25 °C and 30 °C under otherwise standard conditions. PCR was used to confirm the presence of *Acanthamoeba* DNA in positive samples. The findings showed that *Acanthamoeba* grew more rapidly at 30 °C than 25 °C, allowing improved microscopic detection and identification at the former temperature. This investigation shows clearly that the diagnostic sensitivity of an *in vitro* based culture system is temperature-dependent.

Keywords: *Acanthamoeba* spp; keratitis; cultivation temperature; free-living amoebae; *Acanthamoeba* diagnosis.

Introduction

Acanthamoebae are ubiquitous unicellular protozoa that occur in various environments including water, air and soil (Stockman et al. 1992; DeJonckheere 2006; Thomas et al. 2008). It is well documented that under certain conditions, *Acanthamoeba* spp. can cause serious human infections such as *Acanthamoeba* keratitis (AK), especially in contact lens wearers and granulomatous amoebic encephalitis (GAE) in immunosuppressed/compromised patients, such as HIV/AIDS and organ transplant recipients (Aksozek et al. 2002; Bouyer et al.2007; Siddiqui et al. 2019). These organisms are known vectors for a range of human pathogens including viruses, yeast, bacteria, and oocysts of the protozoan genus *Cryptosporidium* [Gómez-Couso et al. 2007; Scheid et al. 2012; Lass et al. 2017).

Of the different diagnostic methods developed to detect *Acanthamoeba*, the most common are based on microscopic examination, the association between culture of the sample, and the polymerase chain reaction (PCR). Nowadays, its diagnosis is usually based on this latter test (Marciano-Cabral and Cabral 2003; Xuan et al. 2017). However, the dependence of the multiplication rate on the cultivation temperature described for a number of protozoas, including amoebae, are poorly defined. In addition, the long duration of the incubation (two weeks) remains a serious limitation (Rahdar et al. 2012). Since if AK is not diagnosed and treated aggressively, and early, extensive ocular damage can occur, requiring enucleating. In addition, granulomatous amoebic encephalitis can develop rapidly, leading to death in ten days (Visvesvara et al. 2007; Orosz et al. 2018). Thus, the diagnosis of this disease is presently not straight forward, and its treatment is very demanding.

The present lack of a standardized method of identification of this free-living amoeba inevitably leads to the need for a long in vitro cultivation period for this organism.

Furthermore, the optimal culture conditions (temperature, pH, nutrients…) for *Acanthamoeba* spp. have been insufficiently explored (Coulon et al. 2012; Delafont et al. 2013). In particular, incubation temperature is a critical factor. Much variation is employed, some researchers choosing a low temperature (25°C) (Jones et al. 1993: Trabelsi et al. 2012; Alves et al. 2015), while others something higher (e.g. 30°C) (Rohr et al. 1998; Qvarnstrom et al. 2006; Khan 2009). In this present study, we have extended our observations on the effect cultivation temperatures have on the culture time *Acanthamoeba* spp. require for morphological identification. For this reasons we are attempting to improve the cultivation method by comparing two temperatures, T1=25°C and T2=30°C.

Materials and Methods:

This study sampled five sites representative of the Rabat region in Morocco: river water, fountain water, tap water, public bath water and coastal ocean water. These areas represent potential habitats for *Acanthamoeba* spp. One hundred and fifty water samples were collected in April through November 2016. Equal numbers of samples were collected from each of the sites mentioned above. The samples were collected in sterile 2-L plastic bottles and were transported directly to the laboratory for parasitological analysis in the Department of General Parasitology, National Institute of Hygiene Rabat, Morocco.

1-culture method for *Acanthamoeba* isolates.

The culture medium used was non-nutritive agar inoculated with heat-treated (60°C for 1 hour) *Escherichia coli* ATCC 25922. Every sample was filtered twice through nitrocellulose membrane filters of a 3-µm pore size (*Acanthamoeba* is 10-25 µm in diameter). The volume of water filtered was based on the amount of material suspended in the samples: 1 L for clean easily filtered water (Fountains, Public bath water and tap

water) and 500 mL for turbid water (Sea and Bouregreg River) which was more difficult to filter. After filtration, the membranes were separately deposited face down on the surface of 1% non-nutrient agar plates previously seeded with 100µl of a heat-killed *Escherichia coli* ATCC 25922 suspension. All culture medium was previously prepared under the same conditions and stored at temperature 4°C for four weeks. For each sample filtered, two Petri dishes were incubated at T2 = 30°C and T1 = 25°C for two weeks and checked daily under an inverted microscope. Each Petri dish was maintained outside of incubator at room temperature for 10±2 minute pre daily during microscopic observations. Cultures were determined as positive for amoebic growth based on morphological characteristics, i.e. the presence trophozoides and cysts, particularly two layered cell wall cysts. Two different temperatures were used to evaluate the influence of temperature on the time required for microscopic identification of *Acanthamoeba*. All positive Petri dish cultures were confirmed using a conventional PCR technique.

2-PCR protocol for *Acanthamoeba* isolates

For DNA extraction, frozen samples were thawed at 22±2 °C. DNA was extracted from *Acanthamoeba* spp. using the following protocol. The cysts of the amoebae and their possible migration traces on Petri dishes were retrieved and suspended in 300 µL lysis buffer (0.1 M Tris-HCL, 0.1 M EDTA, 0.1 M NaCl, 2% SDS). Multiple steps were used to lyse the cellulosic *Acanthamoeba* cyst walls. Five cycles of thermal shock were used, alternating with 1 min of incubation at 95 °C and 1 min in a bath of liquid nitrogen, mechanical lysis by glass beads, and enzymatic digestion with proteinase K for 1 h at 37 °C. Subsequently, the genomic DNA was extracted using a BIOLINE kit according to the supplier's recommendations. The purity of extracted DNA from each sample was determined by absorbance (NanoDrop) at 280 nm. For molecular identification at the genus level, PCR was performed using 18S rRNA gene primers: forward primer (ACA-

F (5′…3′): TGG CAG CGC GAG GAC TAG GG), and reverse primer (ACA-R (3′…5′): ACC GCA CCG ATG GTG GTG TTT) [15]. The amplification was carried out in a PCR thermal cycler. The programme included initial denaturation at 95 °C for 5 min followed by 35 thermal cycles. Each cycle consisted of denaturation at 95 °C for 1 min, annealing at 63 °C for 1 min, and extension at 72 °C for 1 min, and final elongation at 72 °C for 10 min. The PCR products were checked by 1% agar electrophoresis, and the amplified fragments were visualized using ethidium bromide (Schroeder et al. 2001).

Results

Among 150 environmental samples collected for this analysis (Table 1), 20 (13.33 %) were identified as positive for *Acanthamoeba* spp. based on the detection of two-layered cell wall cysts under an inverted microscope (*400). PCR assays with primers specific for *Acanthamoeba* spp. confirmed that 18 of these 20 samples were true positives (Fig. 1 & Fig. 2). Notably, incubation conditions impacted the time required for the microscopic identification of *Acanthamoeba* based on morphology. In this study, we compared two incubation temperatures, 25 °C and 30 °C. When the 18 positive isolates were incubated at 30 °C, the first *Acanthamoeba* were detected by microscopy after five days of incubation (Fig. 1), with the highest number of positive samples (11) observed after six days. In contrast, when samples were incubated at 25°C, seven days were required to detect the first *Acanthamoeba*, with the highest number of positive samples detected after eight days of incubation (Fig. 1). When incubated at 30 °C, *Acanthamoeba* spp. were detected after five or six days and, based on their properties, the amoeba isolates could be divided into two groups: faster growing isolates that could be detected after five days, and moderately slower growing isolates that could be identified only after six days. At 25 °C, a wider and longer window of detection was

150 evident; the first positive samples were apparent after seven days, but it took up to ten
151 days to detect all *Acanthamoeba*. Table 2 illustrates the effects of incubation
152 temperature on the detection of *Acanthamoeba* isolates in water samples. Incubation at
153 30 °C led to a narrow, two-day window of positive identification of *Acanthamoeba*
154 (days 5 and 6), whereas incubation at a lower temperature resulted in a delayed and
155 extended distribution over four days (days 7 through 10)

156 **Discussion**

157 This study demonstrates that incubation temperature impacts the culture time required
158 for morphological identification *Acanthamoeba* spp. The absence of *Acanthamoeba* in
159 the public baths, tap water and fountains is confirmed the good quality of these waters.
160 *Amoebae* were isolated from samples drawn from the Bouregreg river, and from
161 seawater in the Rabat region. Water from these sources contaminated by *Acanthamoeba*
162 spp represents a public health risk, particularly to immunocompromised/suppressed
163 patients and contact lens users (Bunsuwansakul et al. 2019). Based on morphological
164 observations using an inverted microscope, 20 samples were identified as positive for
165 *Acanthamoeba* spp. and PCR confirmed that 18 of these 20 samples were true positives,
166 results that contrast with some previous studies (Khezri et al. 2016; Lazuana et al.
167 2019). The difference between morphological identification and PCR is probably due
168 to amoebae other than *Acanthamoeba* spp. being present in the sample. Incubation
169 temperature conditions employed by researchers to cultivate *Acanthamoeba* spp. varies
170 considerably. Many studies do not report the exact time frame for morphological
171 identification of isolates of *Acanthamoeba* by microscope. Therefore, lack of a
172 standardized method for culture prior to identification of *Acanthamoeba* spp. can be a
173 main contributor for lack of identifying such an organism that needs a long in-vitro
174 cultivation period. Isolates of *Acanthamoeba* are usually able to resist extreme

175 environmental conditions, such as temperature and pH, and, thus, cultivation was
176 performed at two experimental temperatures: T2 = 30°C and T1 = 25°C (Niederkorn et
177 al. 1999; Fouque et al. 2012). Initial positive isolates could be identified by inverted
178 microscopy after five or seven days when cultivated at T2 and T1, respectively. The
179 comparison of different incubation periods indicated that organisms grow more rapidly
180 at T2 = 30°C than at T1 = 25°C, in agreement with previous studies (Greub et al. 2003;
181 Dykova et al. 2005; Zamfir et al. 2006). However, the final positive sample was seen
182 after ten days of culture at T1, compared to six days when incubated at T2. Ghaderifar
183 and co-workers one month to successfully isolate *Acanthamoeba* spp. when incubating
184 at room temperature. Therefore, the higher temperature was considered as the preferred
185 growth condition for *Acanthamoeba* spp. (Booton et al. 2009; Dart et al. 2009; Leduc
186 et al. 2012). The effect of incubation temperature on time required for morphological
187 identification of *Acanthamoeba* isolates indicates that there is an important difference
188 (four days) between the two temperatures we evaluated. This temperature tolerance of
189 *Acanthamoeba* spp. has also been reported elsewhere (Canals et al. 2014; Pumidonming
190 et al. 2010; Yera et al. 2006). *Acanthamoebae* spp. are known to grow more rapidly at
191 higher temperatures (Jones et al. 1992; Rao and Khan 2000; Huang et al. 2017). The
192 time at which the highest number of positive samples was observed varied markedly
193 between the two cultivation temperatures, being 6 days for T2 and 8 days for T1.
194 Previously, a group showed that *Acanthamoeba* isolates can be cultivated at higher
195 temperature (Lonnen et al. 2005).

196 **Conclusion**

197 Taken together, the results of our study indicate that there is a link between cultivation
198 temperature for *Acanthamoeba* spp. and the time required before the morphological
199 identification of these samples using inverted microscopy. To the best of our

knowledge, this is the first report of the effect of cultivation temperatures on the culture time required for morphological identification of isolates of *Acanthamoeba*. Our observations suggest maintaining cultures at a temperature of 30ºC for a period of six days for the initial in-vitro cultivation of *Acanthamoeba* isolates.

Ethical Standards

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Conflict of Interest: the authors declares that he has no conflict of interest

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Figures

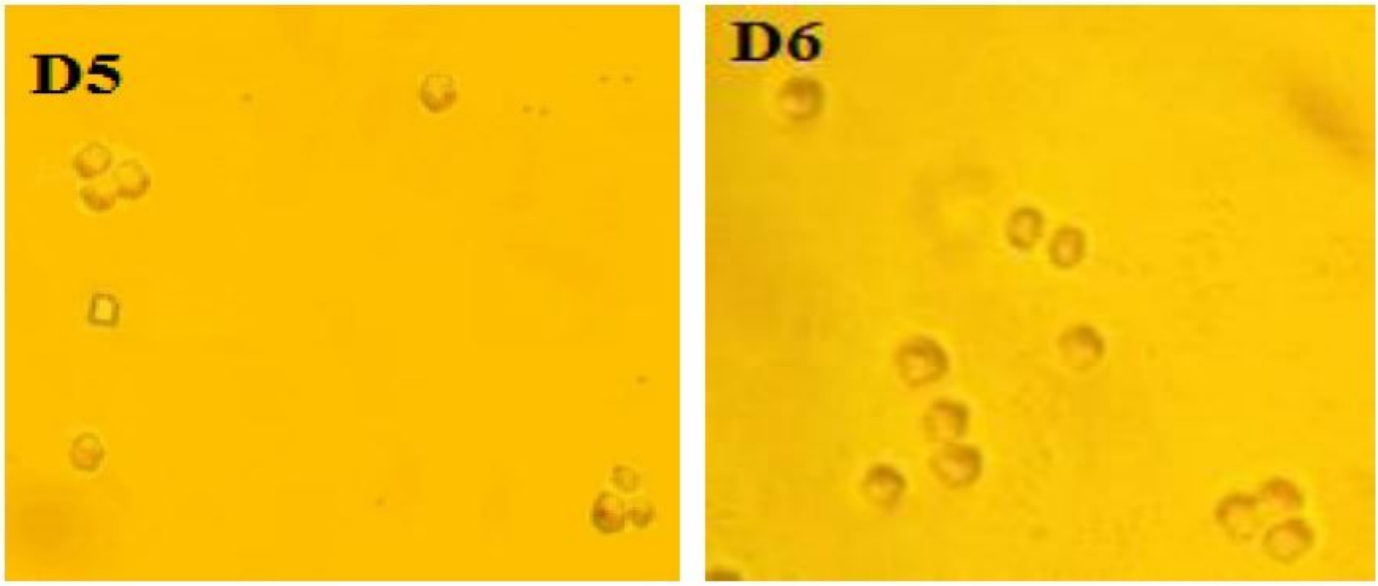

Figure 1

Inverted microscope (*400) results of Non-Nutrient agar culture positive for Acanthamoeba spp incubation temperature at T2. (D5) representative cysts forms of Acanthamoeba spp sample from river water after five days incubation temperature at 30°C. (D6) Acanthamoeba spp cysts sample represent seawater after six days incubation at 30°C.

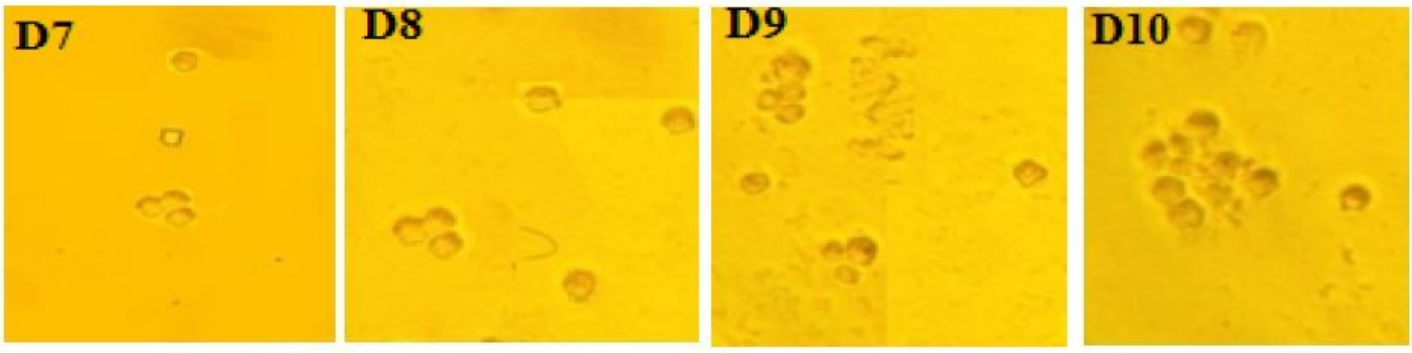

Figure 2

Light inverted microscopy *400 of Acanthamoeba spp. cysts (D7-D10) in co-culture with attenuated Escherichia coli and incubation temperature at T1. The cysts of Acanthamoeba spp, obtained after seven days incubation (D7) at 25°C represent sample form seawater and the sample observed positive at 25°C between D8-D10 represent the sample collected from river.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- table.pdf