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FACULDADE DE FARMÁCIA
DISCIPLINA DE TRABALHO DE CONCLUSÃO DE
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stationary eye washes stations in a public
university from Porto Alegre- Brazil.**

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EM ANEXO.**

The presence of *Acanthamoeba* in portable and stationary eye washes stations in a public university from Porto Alegre- Brazil.

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ABSTRACT

Acanthamoeba is a free-living amoeba (FLA) genus widely distributed in the natural and artificial environments, presenting a great medical and environmental importance. These amoebae are opportunistic pathogens, causing keratitis in healthy people or encephalitis, mainly in immunosuppressed people. The portable and stationary eye-wash stations are equipments required for work environments that may expose employees to harmful chemicals. The purpose of this study is to determine the *Acanthamoeba* genus in FLA isolated from portable and stationary eye wash station, using the PCR technique. These equipment are located in a public university in Porto Alegre, southern Brazil. A total of 74 samples were collected from biofilm (37) and water (37), using sterile swabs and flask, respectively. After processing, the samples were inoculated in non-nutrient agar 1.5%. Of the 74 collected samples, 58 were positive for FLA, but only 38 of these were submitted to molecular identification of the *Acanthamoeba* genus, because some samples which were initially positive for FLA, were lost due to the difficulty of decontamination of microorganisms like fungi. The molecular identification were performed PCR to amplification of the 18S rDNA gene. The PCR products were analyzed and 37 samples were confirmed as belonged to *Acanthamoeba* genus (19 from biofilm, and 18 from water). The *Acanthamoeba* prevalence is reported in several sources of treated water, such as swimming pools and drinking water, and biofilms. The presence of this protozoa in eye wash stations provides risk to their users, since these equipments are used in case of ocular accident, which can cause injury allowing the input of microorganism from water, or biofilm.

1. Introduction

Stationary and portable eyewash stations which go unused for months or years may represent a reservoir of bacteria, protozoa and fungi (Paszko-Kolva, et al., 1991). Some genus of FLA, including *Acanthamoeba spp.*, has been isolated from this equipment (Tyndall, et al., 1987). This FLA genus is an opportunistic protist that is ubiquitously distributed in the environment (Siddiqui and Khan, 2012), and it has been isolated from several sources, such as, swimming pools, tap water, and biofilms from hospital environment and contact lens storages (Carlesso, et al., 2007; Pens, et al., 2008; Caumo, et al., 2009; Winck, et al., 2011).

Acanthamoeba has two stages in its life cycle, a vegetative trophozoite stage, and a dormant and resistant cyst stage. During the trophozoite stage, it actively feed on bacteria, algae, yeasts or small organic particles, and many food vacuoles can be seen in the cytoplasm of the cell. The trophozoites exhibit spine-like structures on their surface known as acanthopodia. This structure is most likely of importance in adhesion to surface, cellular movements or capture prey. *Acanthamoeba* can be maintained in the trophozoite stage with an abundant food supply, neutral pH, appropriate temperature and osmolarity. However, harsh conditions like, lack of food, increased or hypo-osmolarity, extremes in temperatures and pH, induce the transformation of trophozoites into the cyst stage. During the encystment stage, the trophozoite condense itself into a rounded structure, which mature into a double-walled cyst with the outer wall serving only as a shell to help the parasite survive hostile conditions (Khan, 2006). Several studies report that cysts can remain viable for several years while maintaining their pathogenicity, thus presenting a role in the transmission of *Acanthamoeba* infections (Mazur, et al., 1995). The identification of the genus *Acanthamoeba* is usually based on morphology of the trophozoites and cysts, especially on the double wall of the cysts. *Acanthamoeba* isolates can be separated into distinct morphological groups known simply as I, II and III according Pussard and Pons, 1977. Although their taxonomy and classification, are increasingly being revised according to the results of molecular analysis (Caumo, et al., 2009).

The *Acanthamoeba* genus can behave as a “trojan horse” of the microbial world. Many isolates harbor endosymbionts which may include viruses, yeast, protists and bacteria, some of which are potential human pathogens. It is suggested that such

interactions may help transmit microbial endosymbionts to the susceptible hosts and/or endosymbionts may contribute to the pathogenicity of *Acanthamoeba* (Khan, 2006; Greub and Raoult, 2004).

Acanthamoeba cause two well-recognized diseases that are major problems in human health: granulomatous amoebic encephalitis (GAE), which is limited typically to immunocompromised patients, and painful keratitis that can result in blindness. Most cases of *Acanthamoeba* keratitis (AK) are associated with the use of contact lenses due to multifactorial process involving contact lens wear for extended periods of time, lack of personal hygiene, inappropriate cleaning of contact lenses, biofilms formation on contact lenses, and exposures to contaminated water (Khan, 2006). In other cases, the risk of eye infections reportedly increases when eyes are damaged by foreign materials or contaminated water, or when seemingly minor injuries are ignored (Bier and Sawyer, 1990). Though AK is very much associated with contact lenses wear, there are many case reports of AK in non-contact lens wearers (Hirano and Sai, 1999; Sharma, et al., 2004; Syam, et al., 2005; Ertabaklar, et al., 2007; Menghi, et al., 2012). These cases are very important to the disease management, because the non-relationship with lens wear may suggest another type of infection, delayed diagnosis and disease evolution, and in many cases, the corneal scrapings shows negative results for *Acanthamoeba* (Chynn, et al., 1995; Sharma, et al., 2004; Menghi, et al., 2012).

The purpose of this study was to isolate FLA from portable and stationary eye wash station, from a public university from Porto Alegre-Brazil, and identify the *Acanthamoeba* genus using the PCR technique, as well as to discuss the sanitary quality of this equipments.

2. Materials and Methods

2.1. Samples collection

A total of 74 samples -37 of water and 37 of biofilm- were collected between May and June 2010, from 6 locations in a public university in the city of Porto Alegre, RS, Brazil which were: Departments of Physiology, Biochemistry, Zoology, Ecology, Chemical Engineering, and Pharmacy College. Among the 37 eye washes collected, 31 were fixed, and 6 were portables.

The biofilms samples were collected using sterile swabs directly in the water outlet from the eyewashes equipment and the water (50 mL) was collected in sterile centrifuge tubes, by the operation of the equipments.

2.2. Isolation and cloning of FLA

For the isolation of amoebae, the samples were cultivated in non-nutrient agar (NNA) 1,5%, covered by heat-inactivated *Escherichia coli* (ATCC 25922), incubated at 30°C (De Carli, 2001) for up to 15 days. Each plate was examined daily under a light microscope (at 100X) to check the presence of FLA. All positive samples were cloned by the method of dilution, where only one microorganism is seeded in each isolate.

2.3. Morphological identification of FLA

The morphological study was done by the observation of the trophozoite and cyst structures like acanthopodia, contractile vacuoles, nucleus and perinuclear halo, and double wall of the cysts. This analysis was made by the observation of the amoebae in cultive, fixation by sodium acetate, acetic acid and formalin (SAF), followed by trichrome stained (Yang and Scholten, 1977; Garcia and Bruckner, 1993).

2.4. Molecular identification of *Acanthamoeba* isolates

DNA extractions were performed using 3.0 mL of the monoxenic culture containing 10^5 - 10^6 trophozoites, according to Salah and Iciar (1997). Primers JDP1 and JDP2 were used for amplify the ASA.S1 region of the gene (*Rns*) coding for the amoeba's nuclear, small subunit ribosomal RNA. The ASA.S1 fragment allows the specific detection of *Acanthamoeba* since it is discriminating for the genus and can be obtained from all known 18S rDNA (Schroeder, et al., 2001). The polimerase chain reaction was performed according to Booton (2004). Briefly, each reaction was prepared with a total volume of 25 µl, containing 20-30 ng of DNA, 0.2 mM of dNTPs (Ludwig Biotecnologia), 0.4 µM of each oligonucleotide (Invitrogen™), 1.5 mM of MgCl₂, 5× reaction buffer (5× Green/Colorless GoTaq® DNA Polymerase), 1 U of GoTaq® DNA Polymerase (Promega). The PCR conditions included an initial

denaturing in 94 °C for 5 min, followed by 35 cycles in 94 °C for 1 min, 67 °C for 1 min and 72 °C for 1 min. A final extension was performed for 5 min at 72 °C. Amplified products were analyzed by electrophoresis at 70 V in 1.5 % agarose gel (Bioamerica), stained with ethidium bromide and visualized under UV light.

3. Results and discussion

From 75 samples collected, 58 were positive for FLA, and 38 were identified as belonging to the genus *Acanthamoeba* according to the morphological characteristics of their cysts and trophozoites (Pussard and Pons, 1977; Page, 1988) (figure 1), and 37 were confirmed through the PCR technique. This result shows that just the morphological characterization is not sufficient to determine the genus. Among the 37 eye washes analyzed, 31 presented FLA, and 25 (67,6%) presented *Acanthamoeba*, being 18 for water samples, 19 for biofilms samples, and 12 for both (table 1). These results are consistent with literature reports of the isolation of FLA from eye wash station, according to Bier and Sawyer (1990), which isolated FLA from 31 (54,4%) of the 57 water samples from eye washes. These results also agree with the findings of Tyndall, et al., (1987), that isolated FLA from 56 (43,1%) of 130 collections from water. Bowman et al., (1996) found 87% (13 of 15) of the eye wash contaminated for amoeba before flushing, with an average concentration of 168 amoebae/100 mL, and bacteria were detected in all stations, prior to flushing.

In our study, from 31 fixed eye washes analyzed, 24 (77,4%) presented positivity for the genus, whereas one (16,6%), from 6 portables eye washes, presented positivity. The difference between these results may be related to greater exposure of the stationary to external contaminants, due to greater surface contact with environmental microorganisms, than the portable, which has less exposure, and is closed equipment. Furthermore, probably the portable are cleaned more frequently than the stationary eye washes. This greater exposure of the fixed eye washes was confirmed by loss of some samples, which were initially positive for FLA, due to the difficulty of decontamination of microorganisms like fungi.

In conclusion, AK has been recognized as a significant ocular microbial infection. The risk of eye infections reportedly increases when non sterile solutions are used to clean lenses, when eyes are damaged by foreign materials or contaminated water, or when seemingly minor injuries are ignored. Several studies showed that the

corneal trauma is a prerequisite in AK *in vivo*. Nevertheless, corneal trauma followed by exposure to contaminated water, is sufficient, resulting in AK and is the most likely cause of AK in non-contact lens wearers (Niederhorn, et al., 1999; Sharma, et al., 1990; Chang and Soong, 1991; Khan, 2006). However, there are case reports of AK in non-contact-lens wearers, without history of corneal trauma. In some of these cases, the infections were probably caused by the eye contact with contaminated water (Ertabaklar, et al., 2007; Menghi, et al., 2012).

Ocular lesions can be caused by accidents involving chemicals or solid particles, leading to the necessity of using the eye wash. However, when there is not made a frequent maintenance of the equipment, biofilm formation occurs. Thus, these contaminated equipment pose a risk to their users. According Kolva, et al. (1991), practice weekly washing of such devices could prevent biofilm formation, thereby reducing the risk of contamination.

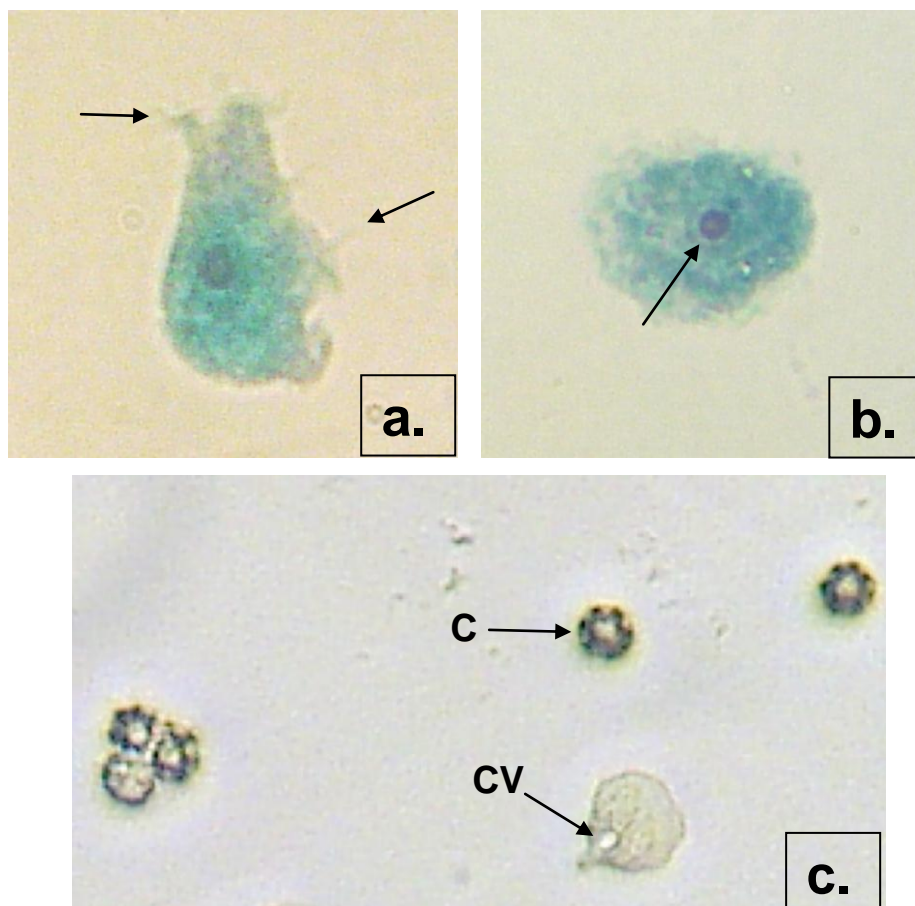


Figure 1: Morphological characteristics observed by Trichromic stain and cultivation in NNA plates. a) Acanthopodia structures; b) Nucleus and perinuclear halo; c) Contractive vacuole (CV), and cyst (C) which presents type I morphology according to Pussard and Pons (1977). IMAGES- Parasitology Laboratory- ICBS/ UFRGS.

Table 1

Positivity of *Acanthamoeba* for each samples type, and equipment type.

Sample type	Number of eye washes		
	Fixed	Portable	Total
Water	17	1	18
Biofilm	18	1	19
Both	11	1	12

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