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A year long study of the presence of free living amoeba in Spain

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ABSTRACT

Free-living amoeba such as *Acanthamoeba* and *Balamuthia mandrillaris* can act as opportunistic parasites on a wide range of vertebrates and they are becoming a serious threat to human health due to the resistance of their cysts to harsh environmental conditions, disinfectants, some water treatment practices and their ubiquitous distribution. This work was carried out in order to study the presence of these free-living amoebae (FLA) and their possible seasonality in a continental-Mediterranean climate in different types of water. For this purpose, a total of 223 water samples were collected during one year from four drinking water treatment plants (DWTP), seven wastewater treatment plants (WWTP) and six locations of influence (LI) on four river basins from Spain. Water samples were concentrated using the IDEXX Filta-Max® system and analyzed by a triplex real time PCR that detects *Acanthamoeba*, *B. mandrillaris* and *Naegleria fowleri*. Agar plates were also seeded for *Acanthamoeba* culture. From the three FLA studied, *N. fowleri* was not detected in any sample while *B. mandrillaris* was found at the entrance of a DWTP; this being, to our knowledge, the first report of these protozoa in water worldwide. On the other hand, the presence of *Acanthamoeba* observed was higher, 94.6% of the studied points were positive by real time PCR and 85.2% by culture, resulting in 99.1% positive for *Acanthamoeba* with both methods. All genetically analyzed *Acanthamoeba* were genotype T4 but nine different T4/DF3 sequences were observed, three of them being described for the first time, assigning new codes. No seasonal distribution of *Acanthamoeba* was found. These facts should serve as a warning to contact lens wearers of the risk of a poor hygiene when handling their contact lenses. It should also serve as a signal to physicians to consider FLA as a possible causative agent of nervous system infections as well as *Acanthamoeba* keratitis due to their high environmental presence shown in this study.

1. Introduction

Acanthamoeba, *Balamuthia madrillaris* and *Naegleria fowleri* are the free-living amoeba (FLA) most commonly related with human diseases. They can be found in different types of water and soil habitats worldwide. *Acanthamoeba* has been isolated from several environments such as freshwater lakes, swimming pools, fresh and salt water, drinking water, contact lens

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washing solutions, ventilation systems, dialysis apparatus and soil among others (Booton et al., 2005; Visvesvara et al., 2007). Both *B. mandrillaris* and *Acanthamoeba* can cause an infection in the Central Nervous System (CNS) called granulomatosus amoebic encephalitis (GAE) as well as lung and skin infections (Visvesvara et al., 2007). *N. fowleri* also causes a CNS infection, primary amoebic meningoencephalitis (PAM), that leads in most cases to the death of the patient within a few days (Visvesvara et al., 2007). On the other hand, *Acanthamoeba* can produce *Acanthamoeba* keratitis (AK), an infection of the cornea that may lead to partial or complete loss of vision (Visvesvara et al., 2007). This infection is becoming more common in industrialized countries due to the increased use of contact lenses and poor handling habits that are some of the risk factors associated with this pathology (Joslin et al., 2007; Verani et al., 2009; Visvesvara et al., 2007). To date, in Spain, only 24 AK cases has been described (Cruz et al., 2004; de Miguel et al., 1999; Lopez et al., 2000; Lorenzo-Morales et al., 2007; 2011; Magnet et al., 2012; Perez Pomata et al., 2006), two of them in the last two years. Granulomatous amoebic encephalitis due to *Acanthamoeba* is a less common infection but worldwide an estimate 400 cases have been described, three of them reported in Spain (Blanco et al., 2012; Gene et al., 2007; Pemán et al., 2008; Seijo Martinez et al., 2000). Environmental studies on FLA epidemiology are scarce in Spain and most of them have been carried out on *Acanthamoeba* on the island of Tenerife, where high concentrations of this amoeba in fresh (59%) and salt water (40%) have been detected (Lorenzo-Morales et al., 2005a,b). Recently, a short study in the central area of Spain, a Continental Mediterranean climate zone, was carried out in drinking water treatment plants (DWTP), wastewater treatment plants (WWTP) and different environmental waters such as reservoirs and rivers. In this study neither *B. mandrillaris* nor *N. fowleri* were detected but a high presence of *Acanthamoeba* was observed (96.4%) (Magnet et al., 2012). There was also a previous study in Spain where *Acanthamoeba* was detected in 2 of 12 finished water samples from three DWTP (Corsaro et al., 2010). Even though the presence of *Acanthamoeba* has been described worldwide and in a great variety of environments, its possible seasonality has not yet been studied in different climates. To our knowledge, there is only one report from a subtropical climate zone, Tulsa (Oklahoma, USA) (John and Howard, 1995), where a year-round study was carried out. In this case, samples were collected monthly during one year from a stock pond, a woodland pond and an ornamental golf-course, and they found that *Acanthamoeba* tends to be isolated in spring and early summer. This peak in *Acanthamoeba* environmental distribution correlates with AK peaks described by Mathers et al. (1998) in a four-year study in a similar climate zone, Iowa (USA).

In the case of *Naegleria* sp. epidemiology, there are a few reports of its presence in Spain; it has been found in a river in the south of Spain (Mascaro et al., 1981) and isolated from the intestinal contents of reptiles (Sesma and Ramos, 1989). To our knowledge there have been no report of *B. mandrillaris* in Spain but one clinical case has been reported in a patient with meningoencephalitis in Portugal (Tavares et al., 2006).

Due to the lack of knowledge about *Acanthamoeba*'s epidemiology in a Continental Mediterranean climate, the aim of the present work was to increase its epidemiomological knowledge during one year sampling (2008) in different types of water.

2. Materials and methods

2.1. Sample collection

Water samples from Castilian Plateau (Spain) were evaluated to characterize the presence of FLA. Four river basins were selected in which 17 sampling points were chosen. These points included four drinking water treatment plants (DWTP), one in each river basin; seven wastewater treatment plants (WWTP), and six locations of influence (LI), one on the edge of a river, two reservoirs and three gauging stations, located upstream and downstream of the water plants. In the DWTP selected, the treatments followed are: preoxidation, precloration, coagulation, flocculation, decantation, sand filtration and disinfection with ozone and/or chloramines. For the WWTP, physicochemical and biological treatments are used with activated sludge. Water samples were obtained following protocol 1623 described by US EPA. For the DWTP, up to 100 L of water were collected from each site (raw water – at the entrance of the plant – and finished water – at the exit of the plant). For WWTP, up to 50 L were collected at both points as above and for the 6 LI up to 50 L. The sampling scheme therefore included the collection of 223 water samples from all the areas under study during the four seasons of 2008, sampling twice in each season (one sample from DWTP 3 could not be included due to processing problems). In all cases water samples were concentrated using IDEXX® Filta Max system following manufacturer's instructions. A total of 7 ml were finally eluted from each concentrated sample and fractioned for different analysis. Samples for molecular analysis were kept at −80 °C.

2.2. Acanthamoeba culture

For *Acanthamoeba* isolation, 80 μl of the concentrated water samples were inoculated onto 2% Neff's saline non-nutrient agar (NNA) plates seeded with heat-killed *Escherichia coli* and incubated at 28 °C. Cultures were monitored daily and subcultured by transferring small pieces of agar containing *Acanthamoeba* to a fresh plate (Magnet et al., 2012).

2.3. Molecular methods

2.3.1. DNA extraction

DNA from water samples were extracted using *DNAeasy® Blood & Tissue Kit* (QUIAGEN, USA) following manufacturer's instructions. These extracted DNA were purified with *QIAquick PCR kit* (Qiagen, USA).

2.3.2. Real-time PCR assay

A triplex real time-PCR designed to simultaneously detect *Acanthamoeba* sp., *B. mandrillaris* and *N. fowleri* was used (Qvarnstrom et al., 2006). PCR amplifications were performed following cycling conditions and structure as described elsewhere (Qvarnstrom et al., 2006) and for every PCR run, positive