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Isolation and Molecular Identification of *Naegleria australiensis* in Irrigation Water of Fuerteventura Island, Spain

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Abstract

Introduction Saline groundwater desalination has recently emerged as an alternative source of irrigation water in arid and semiarid regions due to the gradual reduction in the quantity and quality of conventional water resources for agricultural use. In Fuerteventura Island (Spain), an extremely arid territory in the European Union, brackish water desalination is one of the few available water sources for agricultural production. Very little research has been conducted on the microbiological quality of this water mainly used for irrigation of vegetable crops. Free-living amoebae (FLA) are widely distributed protozoa in the environment and have been isolated from many environmental sources such as dust, soil and water. Among the pathogenic genera included in this group, *Acanthamoeba* spp., *Naegleria fowleri* and *Balamuthia mandrillaris* have been reported to be causative agents of lethal encephalitis, disseminated infections and keratitis. Particularly, *Naegleria fowleri* is a pathogenic FLA species which causes primary amoebic meningoencephalitis (PAM).

Materials and Methods In the present study, the presence of pathogenic FLA strains on desalinated brackish water samples for irrigation has been evaluated during 7 months.

Results From the analysed samples, only one was positive for *Naegleria australiensis.* This is the first report of *Naegleria* spp. in desalinated brackish water for irrigation in Spain.

Keywords Free-living amoeba · *Naegleria australiensis* · Desalinated brackish water · Arid land irrigation

Introduction

Naegleria genus belongs to the free-living amoeba (FLA) group which also includes other opportunistic pathogenic members such as *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Sappinia* spp. and more recently *Vermamoeba* [1, 17, 25, 36].

As FLA members, *Naegleria* species are considered ubiquitous organisms that have been isolated from different environmental sources such as water, soil and air habitats [6, 28]. Within the 47 species belonging to the *Naegleria* genus, only *N. fowleri* has been reported as the aetiological agent of a fatal type of encephalitis known as primary amoeba meningoencephalitis (PAM) [19, 27, 36]. Furthermore, this disease involves a strong inflammation of the brain which is normally revealed as a haemorrhagic-necrotizing meningoencephalitis. Moreover, the most common symptoms of MAP include headache, stiff neck, fever, alteration of mental status, seizure and coma [33].

Naegleria australiensis is widely distributed in Europe, North America, Asia and Oceania [7], and it has been detected in the brain of fish [12]. So far, this *Naegleria* species has only been described as pathogenic to animals (mice) in experimental trials [18]. In that study, *N. australiensis*

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showed cytopathic effects in B-103 rat neuroblastoma cells and exhibited low to moderate pathogenicity for mice.

Fuerteventura Island (Canary Islands, Spain), a UNESCO Biosphere Reserve since 2009, is considered one of the most arid territories in the European Union (mean annual precipitation ≈ 150 mm; evaporation rates ≈ 1800–2000 mm yr^{-1} in evaporimetric tank; [8, 14, 31, 32, 35]). In this environment, agricultural production relies on the use of non-conventional water resources such as recycled urban wastewater, desalinated seawater and desalinated brackish water [9, 10]. Fuerteventura is one of the world's pioneer places in the use of desalinated brackish water for cropping systems. Therefore, the first desalination plant for production of agricultural irrigation water from brackish water was installed even back in the mid-1970s with a small daily capacity of 80 m^3 [37]. Currently, daily production could rise approximately to 9000 m^3 [30]. Because salt removal by reversible electrodialysis or reverse osmosis still entails high energy costs, irrigation with such water quality is economically feasible for only high-value cash crops (e.g. greenhouse vegetables, flowers, etc.). However, technological advances have considerably lowered the production costs of desalinated brackish water (e.g. approximately, 0.2–0.3 € m^{-3} in the Canary Islands; [37], leading to an exponential increase of desalination in many arid and semiarid countries (e.g. Israel, Spain, Australia, United Arab Emirates), where this water is being considered as a supplemental source of irrigation water [2, 9, 20, 22, 23, 29].

Since desalinated brackish water usage in agriculture is still in the early stages, very little research has been conducted on the microbiological quality of these waters and, to our knowledge, this is the first study assessing the presence of pathogenic FLA strains in the water bodies used for crop irrigation.

Materials and Methods

Sample Sites and Culture of FLA

The study was conducted on the volcanic island of Fuerteventura (Canary Islands, Spain), situated in the Atlantic Ocean between 28°45′ and 28°02′ north latitude and 13°49′ and 14°20′ west longitude, and 115 km off the west coast of Africa (Fig. 1). The study site was located on the Pozo Negro experimental farm owned by the local government Cabildo Insular of Fuerteventura. In this farm, the desalinated brackish water is generated from saline groundwater drawn from a depth of 45 m and treated by reverse osmosis at a desalination plant in the experimental farm. Water is stored in top open tanks for a variable period of time until its use for experimental crop irrigation (mainly greenhouse tropical fruit trees and vegetables).

One sample of desalinated brackish water [pH = 7.7/CE (µS/cm) = 701] was collected monthly (January 2018 to July 2018; seven samples) at the stop valves of the experimental fields. The water was collected in 50 mL polyethylene sterile tubes and kept at 4 °C until seeding in the laboratory. Water samples were filtered using a vacuum multiple system and 0.45 µm nitrocellulose filters (Pall, Madrid, Spain).

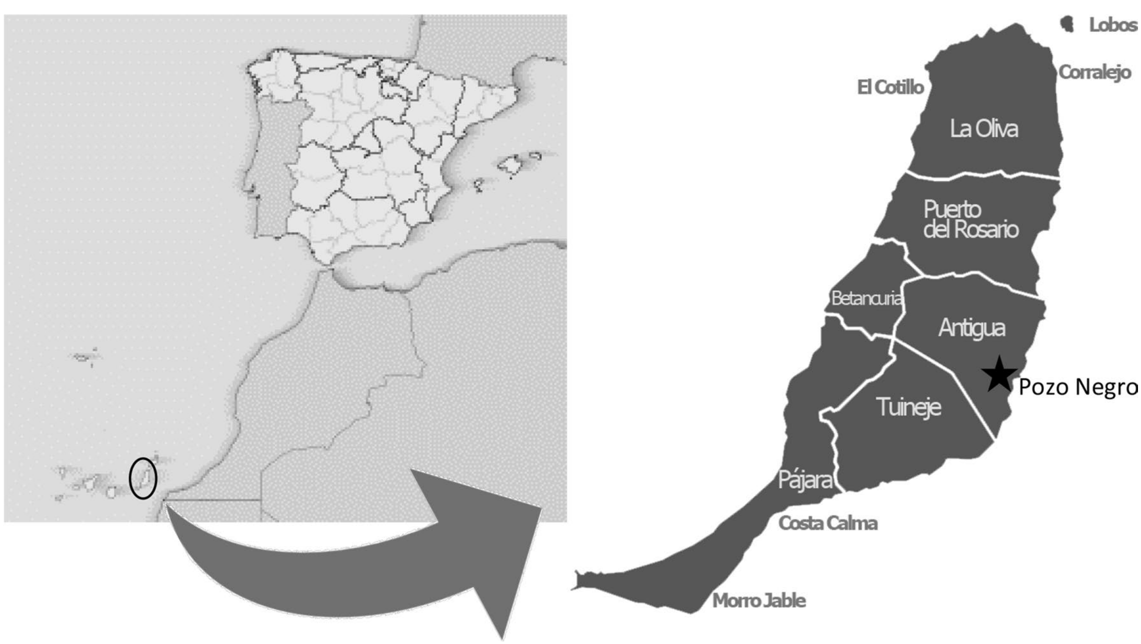

Fig. 1 The island of Fuerteventura and the geographical localization of the experimental farm "Pozo Negro", in Antigua town

Fig. 2 **a** *Naegleria australiensis* trophozoite and cyst (×100); **b** *Naegleria australiensis* cysts (×200); **c** *Naegleria australiensis* trophozoite (×200)

Then, filters were cultured inverted onto 2% non-nutrient agar (NNA) plates with a layer of heat-killed *E. coli* at room temperature and monitored daily for the presence of FLA as previously described in [15, 16] and [26]. The plates suspicious for FLA growth were subcultured until a clean plate was obtained but, unfortunately, axenification was not possible, even using 2% Bacto Casitone (Becton-Dickinson Detroit, Michigan, USA) [13].

DNA Extraction

From the plates with only one morphologic type of FLA, the DNA extraction was carried out using the Maxwell® 16 Tissue DNA purification kit sample cartridge (Promega, Madrid, Spain) following the manufacturer's protocol. Initially, the NNA plates were softly scraped using 4 ml of Page's Amoeba Saline solution (PAS) and the resulting wash was centrifuged (1500 rpm for 10 min) and placed directly into the Maxwell® 16 cartridge. Amoebic genomic DNA yield and purity were determined using the DS-11 Spectrophotometer (DeNovix®, US).

PCR and Molecular Characterization of Isolates

To carry out the molecular identification, PCR analysis was performed using universal FLA primers FLA F 5′- CGC GGTAATTCCAGCTCCAATAGC -3′ and FLA R 5′- CAG GTTAAGGTCTCGTTCGTTAAC -3′ [34]. For all PCR reactions, amplification was performed in a 50 µL mixture containing 80 ng DNA during 40 cycles with denaturation (95 °C, 30 s), annealing (50 °C, 30 s) and primer extension (72 °C, 30 s). After the last cycle, a primer extension was maintained for 7 min at 72 °C and *A. castellanii* Neff ATCC 30010 DNA was used as a positive control in all the PCR reactions. Amplification products were analysed by electrophoresis through a 2% agarose gel and PCR products were sequenced by Macrogen service (Madrid, Spain). The molecular identification was based on sequence analysis of 18S rDNA genus as it has been previously described in comparison with the available FLA DNA sequences in GenBank database [3, 21].

Results and Discussion

Only one of the seven analysed samples (14.3%) was positive for FLA growth in NNA plates and identified as *Naegleria*-like morphologically (Fig. 2). After several subcultures, a clean plate containing only *Naegleria*-like morphology was obtained. To perform a deeper morphological and physiological characterization, a set of clones was prepared for further analysis and was rather uniform. Furthermore, this isolate was able to transform to the flagellated stage and form cysts typical of *Naegleria* genus. Although slight morphological and size differences in trophozoites and cysts of this strain were observed, they were not permanent. Subsequently, molecular characterization of the strain was performed by using PCR/sequencing of the 18S rDNA gene and the strain was confirmed as *Naegleria australiensis* with more than 99% homology when compared to the available *Naegleria australiensis* sequence in Genbank (Sequence ID: U80058.1) [12]. he *Naegleria* strain evaluated in this study was identical and corresponded to the taxonomic criteria set by [24], who summarized several extensive descriptions of *Naegleria* spp. Amoebic trophozoites were able to grow at 37 °C and transform into flagellated swimming stages within 1 h to 1.5 h after the agar plate culture was overlaid with water. The DNA sequence obtained in this study has 99% identity to the *N. australiensis* strain CB2B/I obtained by [12], from the brain of a catfish (a *Clarias macrocephalus* × *gariepinus* hybrid) from a fish farm in Thailand. Strain

CB2B/I was also able to grow at 37 °C (but not at higher temperatures).

Detection of *Naegleria australiensis* in the water could indicate that disinfection processes applied to this water are not adequate for reaching a high biological quality that allow its use in irrigation of any kind of crops (e.g. vegetables consumed without cooking). On the other hand, storage of water in air opened tanks could be an inappropriate management strategy, since several contamination vectors such as dust, rain water or small animal (birds, rodents) waste could be potential sources of FLA. It has been reported that *Naegleria* species such as *N. fowleri* and *N. australiensis* are able to grow at high temperature and saline environments [4, 5, 27]. High temperature can be reached in the storage tanks due to the extreme climatic conditions in Fuerteventura Island (e.g. in summer average temperature 24 °C, average radiation 23.1 M J m^{-2} day^{-1} and average wind speed 3.6 m s^{-1}; [11]). Therefore, it is important to highlight these environmental circumstances, to prevent the proliferation of *Naegleria* species. Furthermore, an additional study assessing FLA presence in desalinated brackish water before and after storage is being carried out by this research group.

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