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Naegleria australiensis isolated from a wastewater treatment station in Santiago Island, Cape Verde

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ABSTRACT

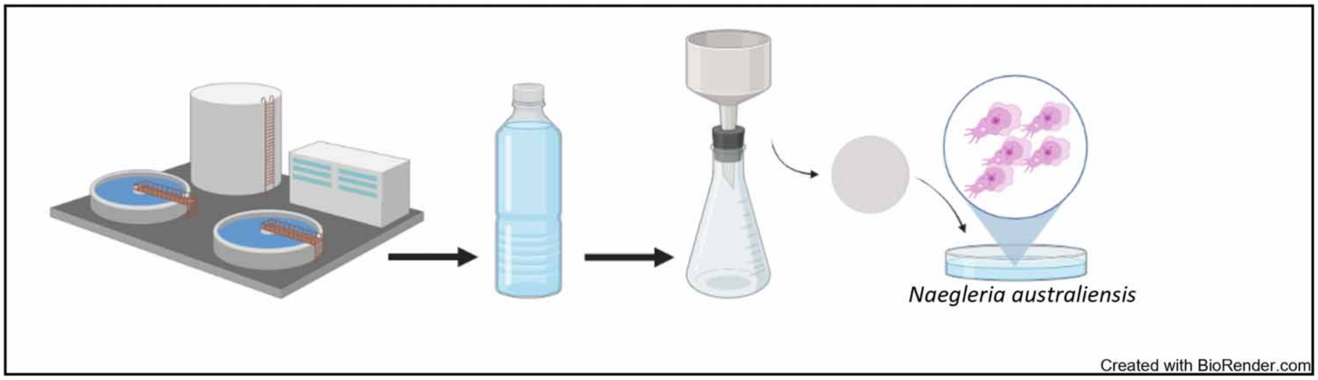
Despite the *Naegleria* genus being isolated from different natural environments such as water, soil, and air, not all *Naegleria* species are capable of causing infections in humans, and they are capable of completing their life cycle in environmental niches. However, the presence of this genus may suggest the existence of one of the highly pathogenic free-living amoeba (FLA) species: *Naegleria fowleri* or the brain-eating amoeba. This facultative parasitic protozoon represents a risk to public health, mainly related to domestic and agricultural waters. In this research, our main objective was to determine the existence of pathogenic protozoa in the Santa Cruz wastewater treatment plant, Santiago Island. Using 5 L of water we confirmed the presence of potentially pathogenic *Naegleria australiensis*, being the first report on *Naegleria* species in Cape Verde. This fact demonstrates the low efficiency in the treatment of wastewater and, consequently, a potential threat to public health. Nevertheless, more studies will be needed for the prevention and control of possible infections in this Macaronesian country.

Key words: Cape Verde, Macaronesia, *Naegleria*, wastewater, water

HIGHLIGHTS

- This is the first report on potentially pathogenic *Naegleria* species in Cape Verde.
- The wastewater treatment stations are a vital resource for the improvement of water use in several arid countries.
- Due to climate change, surface water temperatures are rising, being a susceptible niche to be colonized by pathogenic protozoa.
- The environmental presence of these species represents an important public health concern.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Macaronesia is a world region made up of five North Atlantic archipelagos. They are the islands of volcanic origin, from north to south: Azores (Portugal), Madeira (Portugal), Savage Islands (Portugal), Canary Islands (Spain), and Cape Verde. These island systems share common characteristics from a botanical, climatological, and geological point of view (Ben-Menni Schuler *et al.* 2021). Cape Verde is made up of 10 large islands and five smaller ones (Figure 1). The Windward Islands include Santo Antao, São Vicente, Santa Luzia (uninhabited), São Nicolau, Sal, and Boavista. The Sotavento ones include Maio, Santiago, Fogo, and Brava. They are located 450 km from the coast of Senegal and 1,500 km from the Canary Islands. Santiago is the biggest island of Cape Verde, and its capital, Praia, is in the southeast of the island. Santiago has had a strong influence on the history of the archipelago. Currently, more than half of the Cape Verdeans live on this island. With an area of 990 km^2, this is probably the most diverse island in terms of its landscape. You can find fine sandy beaches, mighty mountains, and dry steppe areas, as well as fertile valleys and plateaus. Even though the hygienic conditions in Cape Verde have improved considerably over recent years, the authorities recommend abstaining from eating unpeeled fruits or drinking tap water (Anon 2022).

Like other Saharan countries, Cape Verde has two distinct seasons: the dry season from December to June and the wet season from August to October (Shahidian *et al.* 2015). More than 75% of the average annual rainfall occurs in the months of August and September (e-Geo & Universidade Santiago Assomada Cabo Verde 2009). The rainy period lasts an average of 15–25 days in arid areas and 45–55 days in semi-arid areas. July and November are considered transition months (Shahidian *et al.* 2015). Despite the low average annual rainfall values, torrential rains concentrated in just a few hours are common in the wettest years. These rains, for reasons related to physiography and land use, run off very quickly, causing floods that overflow the riverbeds and wash away crops, animals, and buildings. This situation contrasts with years in which there is practically no rainfall at all throughout the territory (e-Geo & Universidade Santiago Assomada Cabo Verde 2009). The main Santiago water resources come from the subsoil, about 40 hm^3 per year. These resources are complemented to a lesser extent by surface water stored in small dams and by the desalination of seawater, which is increasingly present in the most important cities of the islands, as a source of urban supply (Santamarta 2013).

In order to achieve the objective of economic sustainability and the fight against water scarcity, Cape Verde, as well as other countries, is investing in the construction of infrastructure for the treatment of wastewater from both domestic and industrial origin, a promising option for supplying urban networks and irrigation systems (Nações Unidas Cabo Verde 2022).

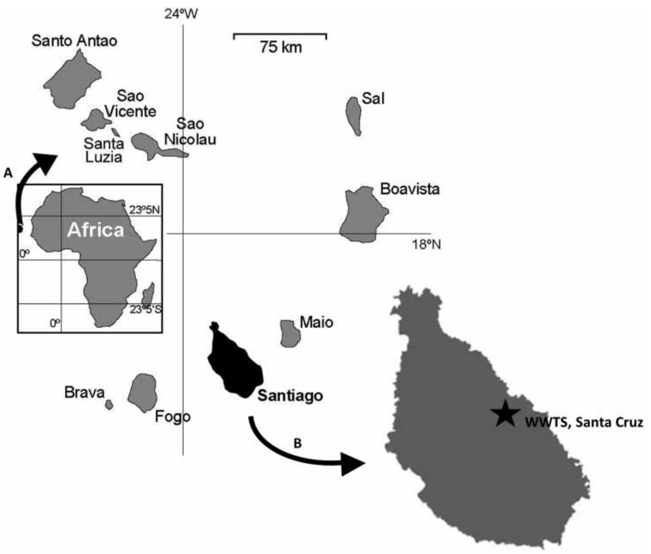


Figure 1 | Representation of Santa Cruz WWTS location in Santiago Island, Cape Verde Archipelago (Modified from Cabral Pinto *et al.* (2015). *Black arrow A* represents the location of the Cape Verde Archipelago related to the African continent. *Black Arrow B and black star* represent the location of the Santa Cruz WWTS related to Santiago Island.

The wastewater treatment stations (WWTSs) are a vital resource for the improvement of agricultural production, guaranteeing additional benefits to this sector, and also contributing to the improvement of sanitary conditions (Mendoza-Grimón *et al.* 2021). There are currently 11 wastewater treatment plants (WWTPs) in Cape Verde, five of them on Santiago Island. The present study has been focused on the WWTS from Santa Cruz, which is a municipality located in the eastern part of the island of Santiago Island. Once treated at this station, this water is used by local farmers to irrigate their crops. Santa Cruz's economy is mainly based on local agriculture, for exporting to larger markets in Praia, Assomada, and Fogo (Mendoza-Grimón *et al.* 2021).

Despite being a critical component of the water cycle, wastewater often receives little social and political attention, neglecting its management, which results in harmful impacts on the environment, economy, and human health. The use of wastewater requires efficient management of operating systems mainly in developing countries to mitigate negative impacts and ensure public health (World Water Assessment Programme (United Nations) n.d.).

Previous studies have reported the presence of free-living amoeba (FLA) in environmental samples from the Cape Verde archipelago, specifically in Santiago Island (Sousa-Ramos *et al.* 2021, 2022). However, as in most countries around the world, in Cape Verde, there is no obligation to declare the presence of FLA in public water sources. Some of these organisms are opportunistic pathogens widely distributed in the environment (Visvesvara *et al.* 2007). FLA such as *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, *Sappinia* spp., and *Vermamoeba vermiformis* are causal agents of severe diseases like encephalitis, epithelial disorders, or keratitis (Visvesvara *et al.* 2007; Scheid 2019). Within the *Naegleria* genus, there are 47 described species (de Jonckheere 2014), and only *N. fowleri* is capable to produce a fatal disease known as primary amoebic meningoencephalitis (PAM) in animals (Bellini *et al.* 2020). On the other side, *N. australiensis* (de Jonckheere *et al.* 1983; Scaglia *et al.* 1989; Latifi *et al.* 2017), *N. philippinensis* (Majid *et al.* 2017), and *N. italica* (de Jonckheere 2005) have been demonstrated as potentially pathogenic species, as they have presented infectivity in mouse models. Therefore, as these four species are capable to thrive not only as free-living organisms but also as parasites, they are considered amphizoic organisms (de Jonckheere *et al.* 1983; Scaglia *et al.* 1989; Latifi *et al.* 2017; Majid *et al.* 2017). *Naegleria* species have been isolated from different environmental sources such as water, soil, and air habitats (de Jonckheere 2011). *N. fowleri, N. australiensis, N. italica, N. lovaniensis,* and *N. philippinensis* have been recognized as thermotolerant organisms, being capable to proliferate at temperatures ranging from 30 to 46 °C (Schuster 2004; de Jonckheere 2014). The previously reported PAM cases present a history of water contact prior to the outcome of encephalitis (Ong *et al.* 2017). Due to climate change and different environmental factors (pH, conductivity, and water availability, among others), surface water temperatures continue to rise, being a susceptible niche to be colonised by these pathogens (Schuster 2004; Martínez-Castillo *et al.* 2016; Maciver *et al.* 2020). Therefore, an investigation of the environmental distribution of these species represents an important public health concern. The present study aims the elucidation of FLA present in tap water from the WWTs in the Santa Cruz municipality from Santiago Island.

2. MATERIALS AND METHODS

2.1. Sample site and culture of FLA

In order to evaluate the presence of potentially pathogenic protozoa in water samples obtained from Santa Cruz WWTS (Santiago Island, 15°08′00″N 23°34′00″O), 10 samples from the same treatment point were collected daily in 0.5-L sterile glass bottles and kept at 4 °C until seeding in the laboratory. The samples were collected from the same point of the WWTP thorough 10 consecutive days. This selected point was chosen because the water coming out of this tap is directly used by the local farmers to irrigate their crops. The water samples were filtered on a vacuum multiple system using a 0.45-μm nitrocellulose membrane (Pall, Madrid, Spain). Once filtered, the membrane was seeded inverted on 2% non-nutrient agar (NNA; Alfa Aesar, Thermo Fisher, Germany) plates, which were incubated at 26 °C and monitored daily to assess the presence of FLA. In order to obtain a monoxenic culture, the plates were cloned by dilution into new NNA plates (Reyes-Batlle *et al.* 2015).

2.2. Molecular characterization of FLA

After morphological identification of FLA growth, genomic DNA was extracted using 4 mL of Page's Amoeba Solution (PAS) to harvest amoeba. Then, this amoebic culture suspension was centrifuged, and the supernatant was discarded. The obtained amoebic pellet was transferred to a DNA purification kit cartridge of the Maxwell® 16 Tissue (Promega, Madrid, Spain) following the manufacturer's instructions. Using the DS-11 spectrophotometer (DeNovix®, Wilmington, NC, USA), the DNA yield and purity were determined.

To amplify the DNA in order to elucidate the FLA species detected, FLA universal primers were used: Ame-f977 5′-GATYAGATACCGTCGTAGTC-3′ and Ame-r1534 5′-TCTAAGRGCATCACAGACCTG-3′ (Liang *et al.* 2010). PCR reactions were performed in a 50 μL mixture, containing 80 ng DNA and the 1 U from the VWR Taq DNA Polymerase with 10× Key Buffer (15 mM $MgCl_2$) kit, with 40 cycles of denaturation (95 °C, 30 s), annealing (62 °C, 30 s) and primer extension (72 °C, 30 s), followed by 2 min of extension (72 °C). The obtained products were analyzed by electrophoresis through a 2% agarose gel with a solution RedSafe™ Nucleic Acid Staining Solution (iNtRON), and sequenced by Macrogen Spain (Madrid, Spain). Species identification was based on sequence homology analysis by comparison with the available DNA sequences in the Genbank database (Reyes-Batlle *et al.* 2019).

2.3. Phylogenetic analyses

Sequences were aligned using Mega-X software program (Kumar *et al.* 2018). The evolutionary relationship was deduced by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei 1993). The tree with the highest log likelihood (−5718.68) is shown and the percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site, involving six nucleotide sequences (KP990616, MK713916, MZ430524, MG699123, MG945025, and the strain isolated in the current study, CVTW1) and a total of 2,022 of nucleotide positions from analyzed sequences in the final dataset.

3. RESULTS

In the current research, 10 water samples from the Santa Cruz WWTP network were evaluated. Only one of them showed FLA-positive growth. After the isolation procedure, a *Naegleria* strain (CVTW1: MW757015) (Figure 2) was detected. After a nucleotide comparison with the available sequence in the GenBank data base, the current sequence presented a ≥95% of homology with previously reported strains of *Naegleria australiensis* recorded in the database. Moreover, as it can be observed in Figure 3, this strain has been clustered close to an *N. australiensis* strain (MG945025.1) isolated from thermal pools in Turkey (unpublished).

4. DISCUSSION

The oceanic location of the Macaronesian archipelagos influences their connections, supply of goods and services, and economy. Therefore, agricultural activity and an important livestock sector dedicated to self-consumption have been developed (Acosta-Dacal *et al.* 2022). Due to its geographical location, Cape Verde is a country vulnerable to climate change. Water scarcity is notorious and directly influences agriculture, which is one of the main activities practiced by most Cape Verdean families (Mendoza-Grimón *et al.* 2021). Due to this fragility, access to treated wastewater becomes an important factor for the sustainable development of the country, considering its multiple benefits (Nan *et al.* 2020). However, due to the lack of

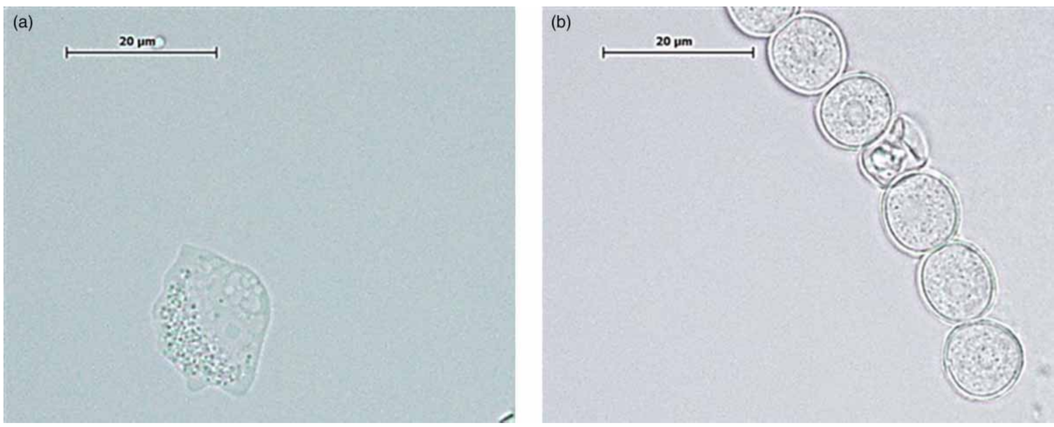


Figure 2 | *Naegleria australiensis* trophozoite (a) and cysts (b) isolated from Santa Cruz, Santiago Island, Cape Verde, in the present study. The images were obtained by the Leica DM1000 LED microscope with the Leica ICC50 W camera.

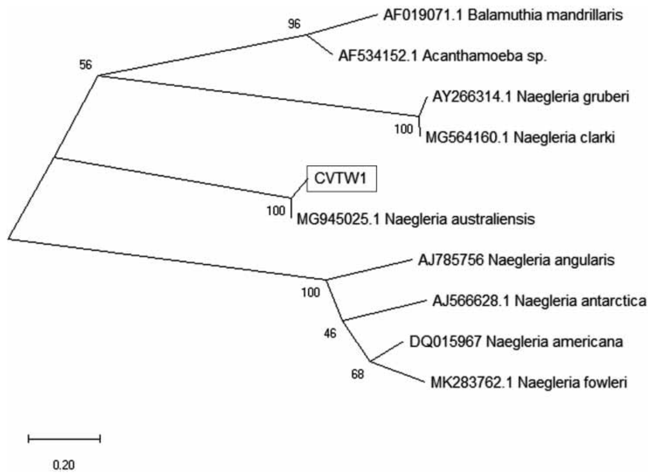


Figure 3 | Evolutionary relationship of *Naegleria australiensis* isolated in the present study (CVTW1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein 1985). The isolate obtained in the present study has been framed in a box.

information/awareness related to the reuse of treated wastewater and their good management, they lead to the informal use of effluents without treatment and without safety control measures (Mendoza-Grimón *et al.* 2021; Marques *et al.* 2022). The use of these water sources in the irrigation of green spaces and in agriculture, carried out by workers without any instruction and protective equipment, increases the risk of serious infections. In Cape Verde, specifically on Santiago Island, this fact could also affect consumers, who supply in the Praia market, if we consider the possibility of not cleaning agricultural products (Monteiro *et al.* 2020; Acosta-Dacal *et al.* 2022).

In the present research, we have demonstrated the presence of *N. australiensis* once through the 10 days of sampling (1/10, 10%). The presence of *Naegleria* species has been reported in WWTPs in Spain (García *et al.* 2011) and Mexico (Ramirez *et al.* 2014). Specifically, *N. australiensis* has been previously reported from an oil refinery wastewater treatment facility in Tehran, Iran (Andalib *et al.* 2022). Moreover, Scaglia and colleagues reported the presence of a pathogenic strain of *N. australiensis* in a spa facility in Italy (Scaglia *et al.* 1983). Like other *Naegleria* species, *N. australienis* can withstand high temperatures ranging from 30 to 42 °C, being capable to adapt to hostile environments including saline environments (Latifi *et al.* 2017). The maximum fixed temperature in Cape Verde, during the year, varies between 25 and 30 °C (Shahidian *et al.* 2015). This temperature range, worsening with global warming, and poor wastewater management, favours the presence of these organisms (Bonilla-Lemus *et al.* 2020; Akbar *et al.* 2021). The presence of *N. australiensis* has been reported in irrigation water from other Macaronesian archipelago, specifically in Fuerteventura Island, from the Canary Islands (Reyes-Batlle *et al.* 2019). However, this is the first report of *Naegleria* species in Cape Verde.

Despite the lack of mandatory reporting of the presence of these FLA as pathogens in water in most countries worldwide, the thermo-tolerance and pathogenicity of *N. australiensis* need to be considered by the local sanitary authorities. Moreover, its presence suggests the possibility of the occurrence of *N. fowleri* in environmental sources in Cape Verde. This raises different facts: (i) the urgent need to develop more research and studies, increasing the sampling size, and (ii) a continuous and real surveillance, to establish a relationship between the presence of these parasites and the wastewater treatment protocol efficacy. Due to the rapid evolution of the reported PAM cases caused by *N. fowleri*, along with the fact that it is a facultative parasite, its presence becomes a public health problem (Abrahams-Sandí *et al.* 2015). Moreover, its difficult diagnosis and high mortality rate result in an even greater socio-economic problem for a country with few resources. It is important to remark that FLA presence contributes to biofilm formation, establishing close relations with several bacteria, viruses, and other protozoa (Preston *et al.* 2001; Khan 2006; Scheid *et al.* 2008). This fact could favour interaction between protozoa and pathogenic bacteria, resulting in serious infections and often neglected deaths (Cateau *et al.* 2014; Schulz-Bohm *et al.* 2017).

Notwithstanding, worldwide several studies have demonstrated the presence of FLA in water supplies, there are few reports of these protozoa in Africa (Baquero *et al.* 2014; Dendana *et al.* 2018; Potgieter *et al.* 2021; Sousa-Ramos *et al.* 2021; van der

Loo *et al.* 2021; Sousa-Ramos *et al.* 2022). Taking into account the variety of species of *Naegleria* and the fact that Cape Verde is an insular country with high temperatures all year round, it becomes a promising country for the dissemination of *Naegleria* species, as well as other thermotolerant and highly pathogenic FLAs such as *V. vermiformis*, detected in other studies carried out in Cape Verde in water samples (Sousa-Ramos *et al.* 2021, 2022).

Since in Santa Cruz the water channels are formed by running water, the risk of contamination by FLA species increases, especially for young people who, in the hottest seasons, seek to cool off by swimming and playing in these channels, despite the local agricultural and industrial products contamination (Gonçalves *et al.* 2015). Thus, water maintenance and quality control protocols should contemplate protozoa presence, not only due to the potential pathogenicity of these microorganisms but also their capacity to transport other pathogenic organisms.

5. CONCLUSIONS

The low efficiency of some WWTPs deteriorates the quality of the existing water on the island of Santiago, allowing the proliferation of pathogens and consequently causing a public health problem. In this research, we describe the identification of *N. australiensis* on Santiago Island with important accentuations for public health in Cape Verde. Although it is not the first time that this species has been reported in all of Macaronesia, its presence implies the need for more studies in the networks of treated water systems, especially in Cape Verde due to its geography and climate. Since studies carried out in Cape Verde have demonstrated the presence of other pathogenic FLA, our research highlights the importance of combining strategies for a deeper investigation throughout the Macaronesia region, focusing on awareness and information for the population, quality treatment for wastewater/sanitation and early detection methods of these pathogens.

AUTHOR CONTRIBUTIONS

D.S.-R. and M.R.-B. conceptualized the study; D.S.-R., M.R.-B. and R.L.R.-E. did methodology; D.S.-R., M.R.-B. and N.K.B. did software analysis; J.E.P. and J.L.-M. validated the study; M.R.-B. and N.K.B. did formal analysis; D.S.-R., M.R.-B. and R.L.R.-E. investigated the study; J.E.P. and J.L.-M. collected resources; D.S.-R. and M.R.-B. did data curation; M.R.-B. and R.L.R.-E. wrote and prepared the original draft; M.R.-B., J.E.P. and J.L.-M. wrote, reviewed, and edited the article; J.E.P. and J.L.-M. visualized the study; J.E.P. and J.L.-M. supervised the study; J.E.P. and J.L.-M. involved in project administration; J.E.P. and J.L.-M acquired funds. All authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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