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First report of *Acanthamoebae* spp. isolation from a volcanic mud spring in the Philippines

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ABSTRACT

Acanthamoebae spp. is considered highly adaptive. The present study aims to establish the occurrence of free-living amoebae, particularly *Acanthamoebae*, to exist in extreme environments such as volcanic mud springs. Fifty surface water samples were collected from mud springs (34 samples), and flat rocks (16 samples) were collected, processed, and cultured. After 14 days of incubation, 32 (64%) plates showed positive amoebic growth. Nineteen (55.8%) of these plates came from the mud spring collection site, while 13 (81.2%) samples are from flat rock sources. DNAs from positive samples were made to react to polymerase chain reaction (PCR) using primer sets JDP1 5′GGCCCAGATCGTTTACCGTGAA-3′ and JDP2 5′TCTCACAAGCTGCTAGGGAGTCA-3′ for cells that resemble *Acanthamoebae*. Sequencing and basic local alignment search tool (BLAST) revealed a 99% similarity of isolates to *Acanthamoebae* spp. Identification of *Acanthamoebae* spp that can survive in higher temperatures is important public health information. The existence of such isolates in the environment has dire health implications, which suggests revisitation of water treatment protocols. Detection of such organisms in environmental sources used for recreational purposes provides information to local and international tourists who frequent them. This will result in the mitigation of potential future infection.

Key words: *Acanthamoebae*, free-living-amoeba, mudspring, Philippines, thermotolerant

HIGHLIGHTS

- First report of *Acanthamoeba* spp. in mud spring sources in the Philippines.
- Isolation of highly thermophilic *Acanthamoeba* spp.
- First FLA surveillance in volcanic mud spring in the Philippines.

INTRODUCTION

Acanthamoebae spp. is the environment's most naturally occurring free-living amoebae (FLA) (Milanez *et al.* 2023). Both the Center for Disease Control and Prevention (CDC) and World Health Organization (WHO) have recognized the public health importance of this organism (WHO 2003; CDC 2019). This is mainly due to the fatal and non-fatal infections it can cause in several mammalian hosts and its capacity to cause outbreaks, as documented by previous reports (Verani *et al.* 2009; Yoder *et al.* 2012; Carnt *et al.* 2018). Several genotypes have been identified to be pathogenic through various *in vitro* and *in vivo* pathogenicity testing (Khan 2003). However, some reports say otherwise and suggest an existence of diversity within the group regarding pathogenic capacity (Howe *et al.* 1997; Milanez *et al.* 2020). For this reason, the isolation of *Acanthamoebae* spp. from unexplored environmental and non-environmental matrices is necessary.

Mud springs are considered extreme habitats for microorganisms (Das *et al.* 2020). Recent studies suggest that prokaryotic and eukaryotic organisms can thrive in such environments (Silva *et al.* 2021). Due to their special growth requirements, these organisms are called extremophiles (Weber *et al.* 2007; Rampelotto 2013). FLA, in particular, are known to thrive in recreational hotsprings and thermal springs and several surveillance studies have been conducted in these environmental

matrices (Fabros *et al.* 2021). Concerning volcanic mudsprings, there is no report of FLA surveillance that has been conducted to the best of our knowledge.

The Mount Makiling Forest Reserve (MFR) is home to Mount Makiling, a dormant volcano in Los Banos, Laguna, on the Island of Luzon, Philippines (ASEAN Center for Biodiversity 2023). The MFR is also a rainforest with diverse fauna biodiversity (Rabor 1977; Mallari *et al.* 2011) and vegetation (Magcale-Macandog *et al.* 2022). Due to this, studies have been directed at creating an information system to catalog different plant and terrestrial animal species (Lapitan *et al.* 2013). Although this is the case, several species potentially present in the MFR are yet to be documented. The MFR is also home to a number of mud springs, hot springs, and flat rock formations due to its geothermal features. Given their thermophilic requirement, these aquatic formations are potential places where FLAs can thrive. To this day, no surveillance studies have been conducted to detect FLAs in the mud spring and flat rock streams in the MFR. This study aims to detect the presence of *Acanthamoebae* spp. in Flatrock streams and the volcanic mud spring in Mount Makiling Forest Reserve.

METHODS

Sampling site

Mount Makiling Forest Reserve (MFR) (Figure 1) is home to diverse aquatic and terrestrial environments. These matrices serve as habitats for microbial, floral, and faunal communities (Magcale-Macandog *et al.* 2021). The mud spring and flat rock formations are popular sites on the MFR trail that local and international tourists frequent. The researchers identified three sampling sites in the mud spring and flat rock streams as represented by upstream and downstream locations (Figure 2). Sampling sites were identified using purposive sampling with consideration of the following: accessibility and evidence of anthropogenic activity. Due to the danger imposed by the mudspring site, it was imperative to limit the sampling criteria to ensure the safety of the researchers who ventured to the site.

Sample collection, processing, and culture

Fifty surface water samples were collected from mud springs (34 samples) and flat rocks (16 samples) and were placed in sterile plastic containers. Sampling sites were identified through sampling points around the area. This was necessary since the area around the volcanic mud spring is an environmentally protected site. Water temperatures from each collection site were noted. After collection, the samples were transported and processed in the Father Lorenzo Rodriguez Laboratory at the University of Santo Tomas. Two-hundred fifty milliliters of water samples were filtered through a 1.2 μm pore size, 47 mm

Figure 1 | The satellite map of Maria Makiling Rainforest Reserve shows the location of the mud spring collection site in the study.

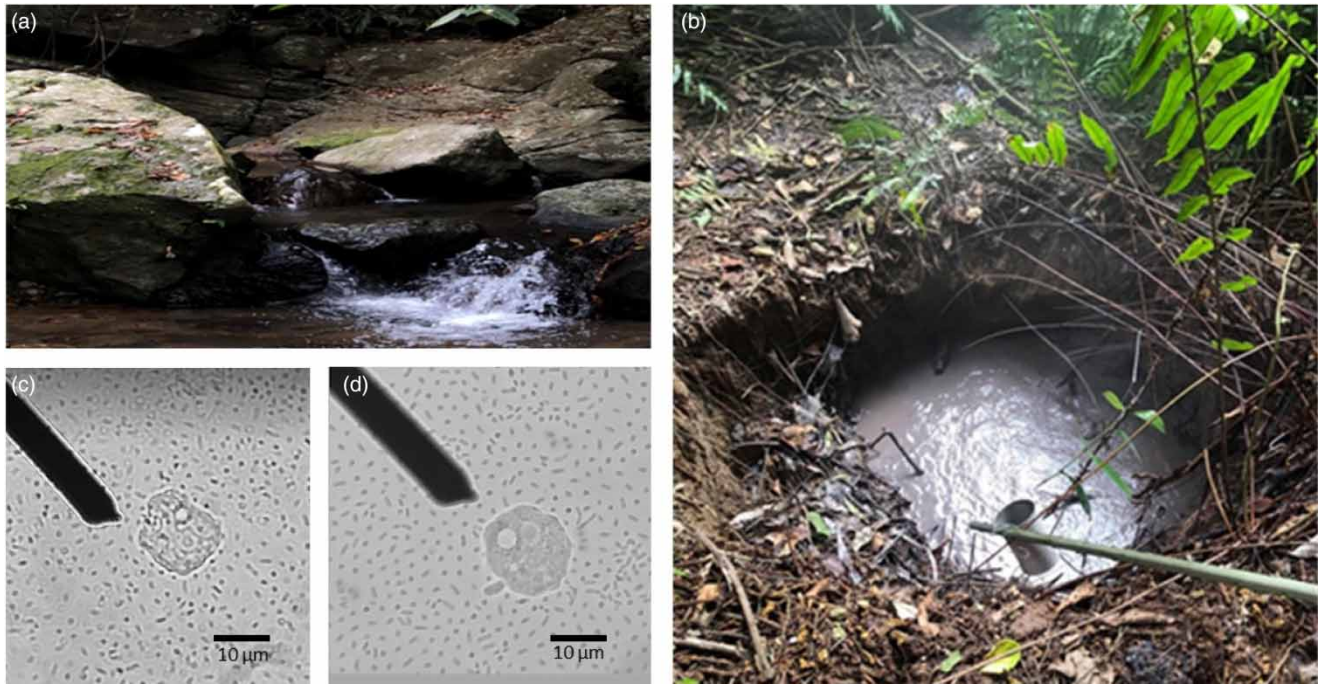

Figure 2 | Photos of collection site in Mt. Makiling Forest Reserve. (a) Flat rock water sources collection site, (b) volcanic mud spring collection site, (c,d) micrographs of amoebic trophozoite stages isolated from the volcanic mud spring in Mt. Makiling. Trophozoite stages exhibit blunt pseudopodia with a single nucleus approximately 11–12 μm. Scale bar set at 10 μm. Magnification 400 ×.

diameter glass microfiber filter (Whatman™, United Kingdom) using a vacuum pump. Filter paper containing sediments was placed on the previously prepared non-nutrient agar (NNA) lawned with *Escherichia coli*, incubated at 30 °C, and microscopically checked daily for amoebic growth for 14 days using a regular compound microscope (Olympus CX23) under 400× magnification. Positive culture plates were subcultured to obtain homogenous growth (Milanez *et al.* 2017).

DNA extraction and molecular analysis of samples

Amoebic cysts and trophozoites were harvested from positive subcultured plates by flooding the agar surface with PAGE amoeba saline stored at 2–8 °C to detach cells on the surface of the agar plate. The surface was gently scraped with a sterile scalpel blade, and approximately 800 μl of fluid suspension was then aspirated and transferred to microcentrifuge tubes. DNAs were extracted using DNeasy PowerWater Kit (Qiagen™, Netherlands) following the manufacturer's protocol. Extracted DNAs are stored at 4 °C or used as a template for PCR amplification immediately. DNAs were made to react to polymerase chain reaction (PCR) (BioRad T100 Thermal Cycler©) using primer sets JDP1 5′GGCCCAGATCG TTTACCGTGAA-3′ and JDP2 5′TCTCACAAGCTGCTAGGGAGTCA-3′ for cells that resemble *Acanthamoebae* spp. PCR conditions were set as follows: 95 °C for 7 min for initial denaturation, 40 cycles of denaturation at 95 °C for 1 min, the annealing temperature of 55 °C for 1 min, extension at 72 °C for 2 min, and a final extension of 72 °C for 15 min (Booton *et al.* 2005). DNA is visualized with 1.5% agarose gel stained with Gel Red® (Biotium, United States). Amplicons were sent to a commercial sequencing company (1st Base, Singapore) for further sequencing. Sequences were aligned using ClustalW of BioEdit with careful visual consideration of gaps and ambiguous sequences.

RESULTS AND DISCUSSION

Our report shows the isolation of *Acanthamoebae* spp. from an extreme environment. This FLA has been considered one of the most adaptive of the FLA group, perhaps because of its ubiquity and widespread prevalence. After 14 days of incubation, 32 (364%) plates showed positive amoebic growth. Nineteen (55.8%) of these plates came from the mud spring collection site (Table 1), while 13 (81.2%) samples are from flat rock sources. Cystic stages showed a characteristic irregular double-walled cyst that measures approximately 8 μm, while trophozoite developmental stages exhibit distinct pseudopodia (Figure 2).

Table 1 | Collection sites in mud spring and the total number of samples collected per site

Collection site	Sample number	Culture	Microscopy	PCR	Blast result
MM1	1	+	+	+	*Acanthamoebae* spp.
	2	+	+	−	
	3	−	N/A	N/A	
	4	+	+	−	
	5	−	N/A	N/A	
	6	+	+	−	
MM2	1	+	+	−	
	2	+	+	+	*Acanthamoebae* spp.
	3	+	+	−	
MM3	1	+	+	−	
	2	+	+	−	
	3	+	+	−	
	4	+	+	+	*Acanthamoebae* spp.
MM4	1	−	N/A	N/A	
	2	+	+	−	
	3	+	−	N/A	
	4	+	+	+	*Acanthamoebae* spp.
	5	+	+	−	
	6	+	−	N/A	
MM5	1	+	−	N/A	
	2	+	+	+	*Acanthamoebae* spp.
	3	+	+	−	
	4	+	+	−	
MM6	1	+	+	+	*Acanthamoebae* spp.
	2	−	−	N/A	
	3	+	+	−	
	4	−	N/A	N/A	
MM7	1	−	N/A	N/A	
	2	−	N/A	N/A	
	3	−	N/A	N/A	
	4	−	N/A	N/A	

Table shows culture, microscopy, and PCR results from isolates. Table showing positive isolation of *Acanthamoebae* spp. in six collection sites.

Interestingly, acanthanpodia was not observed from trophozoites seen from all samples. Six samples from the mud spring collection site and one from flat rock sources showed a positive band between 400 and 500 bp using JDP1 and JDP2 primer sets. Sequencing and basic local alignment search tool (BLAST) revealed a 99% similarity of isolates to *Acanthamoebae* spp. and was registered in GenBank with accession number OR447488. Although *Acanthamoebae* spp. have been reported to persist in extreme environments such as desert soils (Rodriguez-Zaragosa *et al.* 2005) and frozen waters (Brown & Cursons 1977), it is important to consider that isolate Mt. Makiling Mudspring in our study was from a water source with a registered temperature of 80 °C. This suggests that some *Acanthamoebae* spp. strains can possibly adapt to extremely high temperatures in the environments. The existence of such isolates in the environment has dire health implications, as *Acanthamoebae* spp. is currently known to persist with temperatures not more than 65 °C of moist heat (Coulon *et al.* 2010). Conversely, certain water treatment protocols to deactivate FLA cystic forms may need to be revisited as current protocols may lack the necessary temperature to decontaminate the water from FLAs. In the case of *in vitro* testing to assess FLA pathogenicity, it may not apply with isolates that exhibit high resistance to temperature such as the isolates in this study. To determine the pathogenic capacity of such isolates improvisation may be needed to conclude the pathogenic effects, especially those from environmental samples. Although there was no direct evidence on the exact reasons for the occurrence of isolate MMM in this study in the volcanic mud spring, we can only hypothesize their existence. One possible reason is the continual exposure of soil isolates to the volcanic mud spring. It should be emphasized that soil surrounding the volcanic mud spring may contain *Acanthamoebae* spp. and due to exposure over time to high temperatures may have adapted

to the environment. Soil runoff from terrestrial sources to the aquatic mud spring may have been possible as well. The continual change in climate where the volcanic mud spring is located may have also been a reason. Although there are no conclusive data that we have gathered to prove all these, it is important to consider that the volcanic mud spring is situated in an environment where constant changes in temperature and precipitation are observed. Having said this, further investigation is necessary to provide justification to this claim.

CONCLUSIONS

Detection of FLAs from environmental sources is considered paramount in disease prevention. This does not only establish the presence of pathogenic organisms but importantly, prevents further potential human encounters which lead to disease causation. Also, surveillance of unexplored environmental matrices may lead to the detection and identification of potential new strains that have the capability to evade established treatment protocols. Here we present preliminary data on the occurrence of a highly thermophilic *Acanthamoebae* spp. isolated from an extreme environment. Further testing on isolate MMM is necessary to determine the pathogenic capacity of the isolate. The detection of such organisms in environmental sources used for recreational purposes provides information to local and international tourists who frequent them. This will result in the mitigation of potential future infection. The authors declare to the best of our knowledge that this is the first isolation of *Acanthamoebae* spp in volcanic mud springs in the Philippines.

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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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