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Detection of *Acanthamoeba* spp. in groundwater sources in a rural area in the Philippines

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ABSTRACT

Research on free-living amoebae (FLA) and its public health implication as an etiologic agent of parasitic infection in humans has recently gained traction in the Philippines. This study aimed to identify potential FLAs in collected groundwater samples from Masinloc, Zambales, Philippines. Fifty-four (54) water samples were collected in 250-mL sterile polyethylene containers by purposive sampling from selected groundwater sources in six (6) barangays of Masinloc. The samples were vacuum filtered through a 1.2-μm pore glass microfiber filter, cultured onto non-nutrient agar (NNA) lawned with *Escherichia coli*, and observed microscopically for amoebic growth for 14 days using light microscopy. Amoebic growth was observed in 11.1% (6/54) of water samples. DNAs from positive samples were extracted and were made to react with polymerase chain reaction using *Acanthamoeba*-specific JDP1 (5′-GGCCCAGATCGTTTACCGTGAA-3′) and JDP2 (5′-TCTCA-CAAGCTGCTAGGGAGTCA-3′) primers, and universal primer Euk A (5′-AACCTGGTTGATCCTGCCAGT-3′) and Euk B (5′-TGATCCTTCTGCA GGTTCACCTAC-3′). The presence of *Acanthamoeba* genotypes T4, T7, and T11 was confirmed using molecular and phylogenetic analysis. Our results confirmed that groundwater sources from two of six sampling sites (33.3%) in Masinloc, Zambales, were contaminated with potentially pathogenic FLAs. Proper identification of risk factors that may cause contamination consequently leads to the implementation of programs that will prevent future infections.

Key words: free-living amoeba, keratitis, Philippines, protozoa, trophozoite, water

HIGHLIGHTS

- First report of *Acanthamoeba astronyxis* in groundwater sources in the Philippines.
- First report of *Acanthamoeba* genotype T4 in groundwater sources in the Philippines.
- Confirmed pathogenic *Acanthamoeba* spp. through thermo-tolerance testing in groundwater.
- First extensive survey of FLAs in groundwater sources in the Philippines.
- Potential risks of FLA occurrence in groundwater sources.

INTRODUCTION

Free-living amoebas (FLAs) are a group of ubiquitous protozoans that thrive in several environmental matrices. According to the World Health Organization (2003), four genera of FLA, namely *Acanthamoeba* spp., *Naegleria* spp., *Balamuthia mandrillaris,* and *Sappinia* spp. are considered medically important due to FLA-related infections (Visvesvara *et al.* 2007; Angelici *et al.* 2021). Among the FLAs, *Acanthamoeba* spp. is known to be widely distributed in the environment. These protozoans can cause both non-fatal and fatal health conditions (Balczun & Scheid 2017; Milanez *et al.* 2020). Several genotypes are known to cause granulomatous amoebic encephalitis (GAE), *Acanthamoeba* keratitis (AK), and disseminated cutaneous infections in both immunocompetent and immunocompromised hosts (Khan 2006). *Acanthamoeba* spp. has been reported to be isolated from a variety of natural and man-made environments such as soil (Cruz & Rivera 2014; Xuan *et al.* 2017; Meighani *et al.* 2018), marine water (Hussein *et al.* 2021), lakes (Ballares *et al.* 2020; Milanez *et al.* 2022), and swimming pools (Esboei *et al.* 2020). Due to their ability to thrive in a plethora of environments, the isolation of these protozoans in major aquatic sources is important to mitigate potential infections due to anthropogenic activities (Masangkay *et al.*

2022). Groundwater is a significant source of potable drinking water worldwide (Carrard *et al.* 2019). The Centers for Disease Control and Prevention (CDC) reported that groundwater, such as those coming from wells, is naturally filtered to some extent (CDC 2022). The lack of knowledge on the maintenance of groundwater sources and their potential biological contaminants may pose a public health threat due to its possible exploitation and contamination of soil-thriving microorganisms that may cause morbid to mortal health conditions (Giordano 2009). This has been validated by recent reports on the simultaneous contamination of protozoan pathogens in well water sources in the Southeast Asian region (Masangkay *et al.* 2022), and of tap water sources as well (Coşkun *et al.* 2013; Üstüntürk-Onan & Walochnik 2018).

In a study conducted in 10 Southeast Asian countries, groundwater is preferred over surface water for drinking and domestic use (Carrard *et al.* 2019). In the Philippines, especially in rural areas, groundwater is the main water source for domestic and irrigation purposes (Inson *et al.* 2021). Groundwater is the most extracted raw material globally and is prone to pollution/contamination (Alsalme *et al.* 2021). Due to the vast availability and accessibility of groundwater, it is overexploited in many parts of the world (Molle *et al.* 2018). Although there are monitoring guidelines currently being implemented to check the quality of the groundwater sources in terms of pH, temperature, electrical conductivity, total dissolved solids, salinity, and static water level (Oppus *et al.* 2020), there appears to be no established protocol for protozoans such as *Acanthamoeba* species. This study aimed to isolate and identify *Acanthamoeba* spp. from groundwater sources in the province of Masinloc, Zambales, Philippines.

METHODS

Study setting

The municipality of Masinloc (158°35′06″93N 119°55′43″86 E) is a coastal town in the province of Zambales, Philippines. It is in the northwestern portion of Zambales in Region III with a total land area of 33,150 ha and 30 km north of Iba, the provincial capital. It has a total population of 49,955 people, with 11,456 households (Municipal Planning and Development Office (MPDO) 2016 Census of Households). Fishing, farming, commerce, and mining are all major industries in this area. It is divided into 13 barangays, namely North and South Poblacion, Baloganon, Collat, Inhobol, Taltal, Bani, Sta. Rita, Tapuac, San Salvador, Bamban, Sto. Rosario, and San Lorenzo (Masangkay *et al.* 2020, 2022). Groundwater is the main source of fresh water for various purposes in Masinloc. Six (6) barangays in Masinloc, Zambales were selected as study sites, namely Barangay Baloganon, Bamban, Inhobol, Sto. Rosario, San Lorenzo, and South Poblacion (Figure 1). These sampling sites were chosen based on freshwater sources (public access wells), road access, and the population's frequency of using groundwater sources from pumps. Eighteen (18) water pumps were further identified from the study sites where groundwater samples were collected (Table 1).

Water sample collection, processing, and culture

A total of 54 (three samples from each water pump source) groundwater samples from six selected barangays were collected in 250-mL sterile polyethylene containers (Table 1). The samples were transported to the laboratory at the Department of Medical Technology, Far Eastern University Manila, Philippines for processing. Samples were vacuum filtered through a 1.2-μm pore size glass microfiber filter. Filters containing sediments were placed sediment-side down on non-nutrient agar (NNA) lawned with live *Escherichia coli* and incubated aerobically at 33 °C for 14 days. NNA plates were examined microscopically for amoebic growth using a compound microscope (Nikon Eclipse E100) under 400× magnification. The agar surface was scanned for the presence of cysts and trophozoites. Positive plates were subcultured to obtain homogenous growth following previously established protocols (Milanez *et al.* 2020). Briefly, approximately 1 cm × 1 cm of agar block from an identified area with abundant growth was cut using a sterile scalpel blade and then placed culture-side down onto a fresh NNA plate lawned with live *E. coli* and was incubated and examined under similar conditions as before. These steps were repeated until a homogenous culture was obtained.

DNA extraction and molecular analysis

Suspected *Acanthamoeba* spp. cysts and trophozoites were harvested from culture plates by flooding the agar surface with cold phosphate-buffered saline solution (pH 7) and gently scraped with a sterile scalpel blade (Milanez *et al.* 2019). The fluid was then aspirated and transferred to microcentrifuge tubes, and DNA was extracted using QIAamp® DNA mini stool kit, following the manufacturer's instructions. Primer pairs JDP1 5′-GGCCCAGATCGTTTACCGTGAA-3′ and JDP2 5′-TCT CAAGCTGCTAGGGGAGTCA-3′ were used to amplify the ASA1.S1 region of the *Acanthamoeba* partial sequence

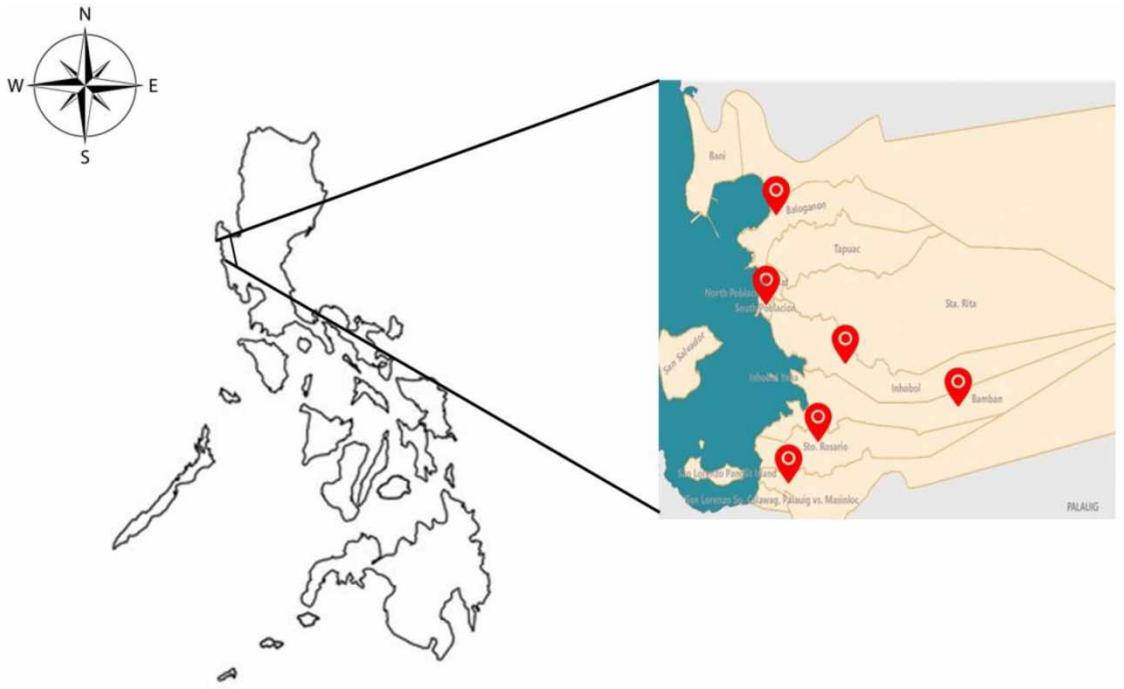

Figure 1 | Map of the Philippines showing the six selected sampling sites for this study. Study sites were identified as pinned location sites, namely Barangay Baloganon, Bamban, Inhobol, and Sto. Rosario and San Lorenzo.

Table 1 | The total number of water pumps and water samples collected at each study site

Barangay	Number of water pumps	Number of samples collected
Baloganon	3	9
Bamban	4	12
Inhobol	3	9
San Lorenzo	2	6
Sto. Rosario	4	12
South Poblacion	2	6
Total	18	54

Note: Three water samples were collected at each water pump.

(Booton *et al.* 2005). Thermal cycling conditions were set at initial denaturation of 95 °C for 7 min, 40 cycles at 95 °C for 1 min, annealing temperature at 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). PCR products were visualized by performing 1.5% agarose gel electrophoresis stained with ethidium bromide. To further detect other FLAs in our samples, gel electrophoresis-negative samples were subjected to another round of PCR using universal eukaryotic primers Euk A (5′-AACCTGGTTGATCCTGCCAGT-3′) and Euk B (5′-TGATCCTTCTGCAGGTTCACCTAC-3′). Thermal cycling conditions for this primer set were set at 94 °C for 5 min initial denaturation, 30 cycles of denaturation at 94 °C for 45 s, annealing temperature of 52 °C for 1 min, extension at 72 °C for 2 min, and a final extension of 72 °C for 7 min (Medlin *et al.* 1988) and visualized through gel electrophoresis as before. *Acanthamoeba* spp. genotypes were identified through DNA sequencing and phylogenetic analysis. PCR amplicons were sent to a commercial sequencing company (Macrogen, Seoul, South Korea). Sequences were aligned using ClustalW of Bio-Edit with careful visual consideration of gaps and ambiguous sequences and were deposited in GenBank. Phylogenetic analysis of isolate sequences along with reference strains from GenBank was performed using the maximum likelihood (ML) tree constructed with 1,000 replicates using MEGA 11.

Thermo-tolerance assay

Thermo-tolerance assay on sequenced isolates was performed following previously established protocols (Walochnik *et al.* 2000). Briefly, 1 μl of cystic stage suspension of isolates was cultured at the center of freshly prepared NNA plates lawned with live *E. coli* and incubated in varying temperatures, i.e. 30, 37, and 40 °C. Amoebic growth was observed for 48 h with emphasis on the outward migration of trophozoites from the point of inoculation.

RESULTS

Culture and microscopy observations

After 14 days of incubation, 6 out of 54 (11.1%) groundwater samples showed positive amoebic growth after microscopic analysis. Positive samples came from two sampling sites, namely Barangay Inhobol (Isolates IN2.1, IN2.2, IN3.1, IN3FLA, IN3.3) and Barangay Sto. Rosario (Isolate SR2.3). Light microscopy revealed double-walled cystic stages ranging from 9 to 11 μm and motile trophozoite exhibiting acanthapodia (Figure 2). The initial morphological classification of amoebic growth as *Acanthamoeba* spp. was based on cyst size and morphology according to Page's established morphological criteria (Page 1967).

Molecular and thermo-tolerance findings

Five (5) positive samples using the primers JDP1 and JDP2 showed a distinct band between the 400 and 500 bp region in the agarose gel electrophoresis, while sample IN3FLA demonstrated a band formation using the primers EukA and EukB. This confirmed the presence of six *Acanthamoeba* spp. isolates in the groundwater samples from two barangays of Masinloc, Zambales. Sequences of isolates IN2, IN2.2, IN3.1, SR3.2, IN3.3, and IN3.3FLA were deposited in GenBank and were assigned with accession numbers OP411026, OP411028, OP393107, OP404258, OP404254, and OP393199, respectively. Sequencing and Basic Local Alignment Search Tool (BLAST) percent similarity of DNA revealed *Acanthamoeba* spp. belonging to genotypes T4, T5, and T15 (Table 2). Further phylogenetic analysis of sequences confirmed the identity of *Acanthamoeba*

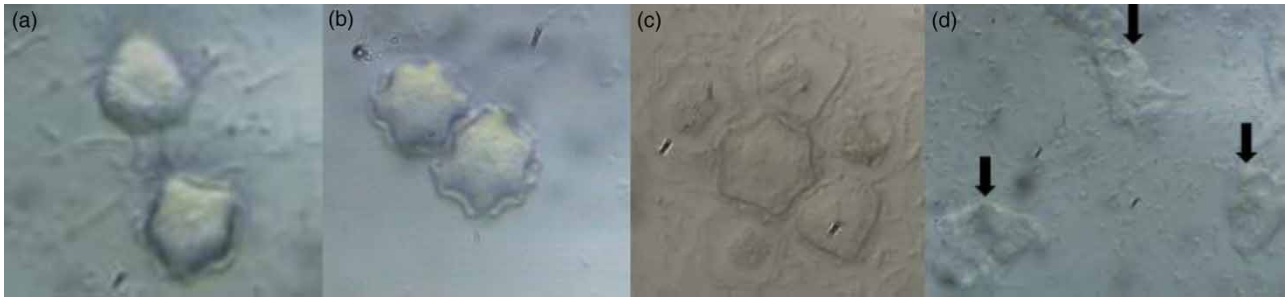

Figure 2 | Photomicrographs of *Acanthamoeba* spp. cystic stages (a–c) and trophozoite (d) isolated from the selected groundwater sources in Masinloc, Zambales showing morphological irregular double wall and acanthapodia, respectively. Magnification: 400 ×.

Table 2 | Initial BLAST results and thermo-tolerance assay results on *Acanthamoeba* spp. isolates from Masinloc, Zambales

Isolate	Accession numbers	Genotype	Percent similarity (%)	Thermo-tolerance assay			Genotype-associated disease (Scheid 2018; CDC 2019)
				33 °C	37 °C	40 °C	
IN2.1	OP411026	T4	99	+++	++	++	Encephalitis, keratitis
IN2.2	OP411028	T4	99	+++	++	++	Encephalitis, keratitis
IN3.1	OP393107	T7	96	+++	++	−	Encephalitis, keratitis
SR3.2	OP404258	T4	100	+++	+	−	Encephalitis, keratitis
IN3.3	OP404254	T11	99	+++	++	−	Encephalitis, keratitis
IN3FLA	OP393199	T11	98	+++	++	−	Encephalitis, keratitis

Note: In vitro pathogenicity test shows that two isolates (IN2.1 and IN2.2) are capable of persistent growth at 40 °C.

astronyxis, *Acanthamoeba hatchetti*, and *Acanthamoeba* genotype T4 (Figure 3). Thermo-tolerance assay demonstrated that two isolates (IN2.1 and IN2.2) are potentially pathogenic with the ability to persist up to 40 °C incubation.

DISCUSSION

The critical contribution of groundwater as a primary source of fresh water for drinking and domestic use in low-income countries is unquestionable (Velis *et al.* 2017). For this reason, achieving access to clean water for all is included in the

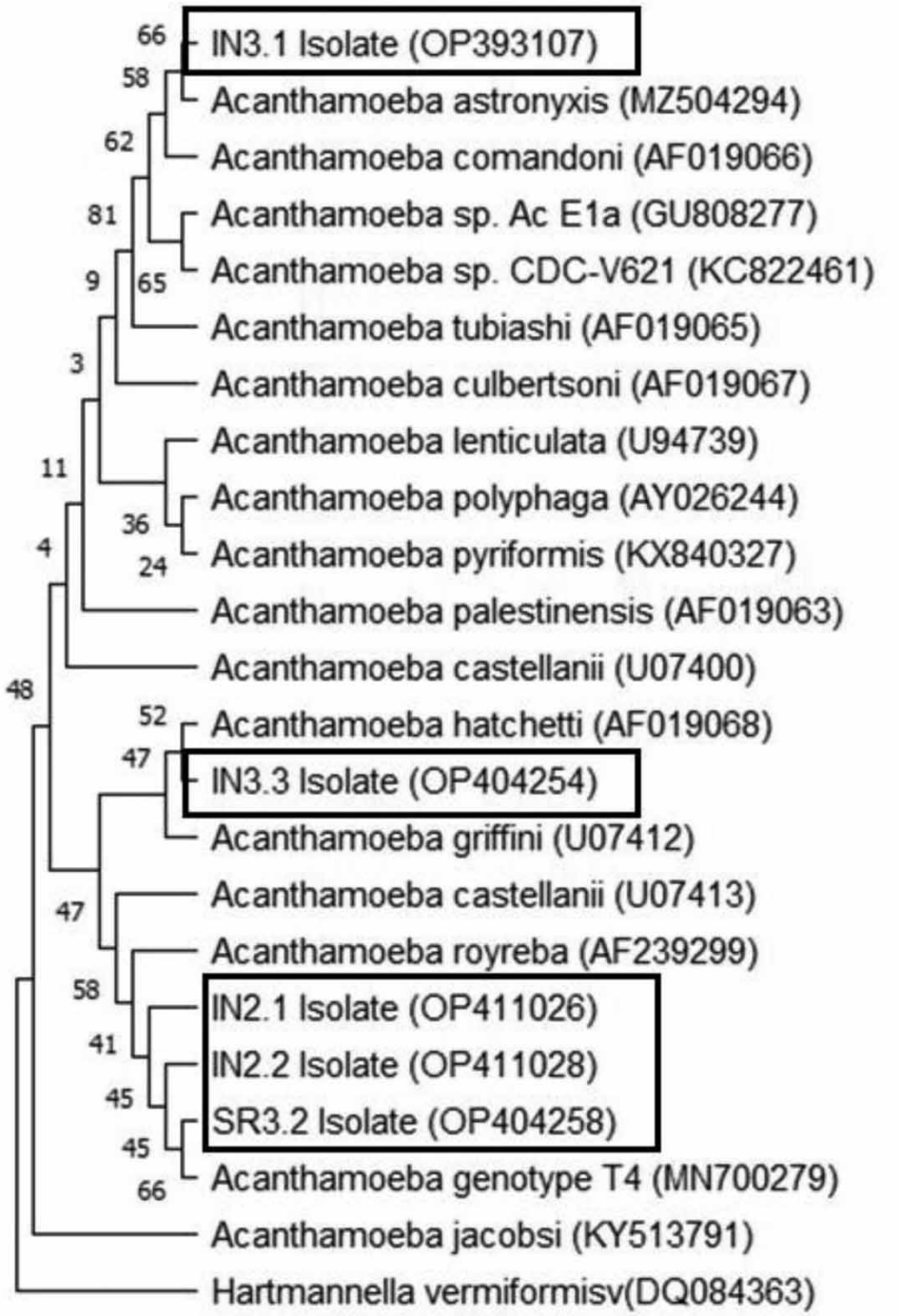

Figure 3 | Maximum likelihood phylogenetic relationships of the partial 18S rRNA sequence of isolates IN3.1, IN3.3, IN2.1, IN2.2, SR3.2 from this study and reference strains of *Acanthamoeba* spp. sequences deposited in GenBank. Accession numbers are in parentheses. Bootstrap value was set at 1,000 replicates. The tree was constructed using the Tamura 3-Parameter model using MEGA 11.

sustainable goals set by the United Nations (United Nations 2022). Although there are several guidelines for the treatment of groundwater, potential contamination due to several environmental and non-environmental factors is unavoidable (Brindha & Schneider 2019). For this reason, continued monitoring for potential adulteration of these sources is paramount to ensure public health and safety. The contamination of groundwater with pathogenic organisms is considered a prelude to a public health catastrophe. Here, we have detected the presence of *Acanthamoeba* spp. in the groundwater sources for drinking and domestic use of the population in Masinloc, Zambales. Although our isolation rate is at 11.1% (6/54) of the total samples from different study sites, it is important to note that the sites were the most frequently used by the population. Moreover, the positivity rate is concurrent with our previous study in well water sources collected during the wet season (Masangkay *et al.* 2022). *Acanthamoeba* spp. has been successfully isolated from soil and dust, as documented by several studies (Cruz & Rivera 2014; Reyes-Batlle *et al.* 2016; Xuan *et al.* 2017). Also, soil runoff and saltwater intrusion have been proposed as contributory factors for *Acanthamoeba* spp. contamination of groundwater sources (Masangkay *et al.* 2020; Milanez *et al.* 2020). Although the exact mechanism of the contamination of the groundwater sources of the current study remains elusive, it is important to note that the environment where the water pumps are installed can be described as lacking cleanliness and the water pumps worn out (Figure 4).

Acanthamoeba spp. causes two important health conditions: non-fatal but morbid ocular infection (AK) and fatal encephalitis (GAE). AK is commonly observed in contact lens wearers (CLWs), with the usual cause of infection being unhygienic cleaning and storage of the lenses (Shimmura-Tomita *et al.* 2018). Nevertheless, non-contact lens wearers (NCLWs) may also contract AK through the exposure of the eye to contaminated water, or trauma with a contaminated object (Singh *et al.* 2020). This was the suspected cause of the AK case of a non-contact lens wearer in the Philippines who used tap water to regularly cleanse the face (Buerano *et al.* 2014). This case provided evidence of the potential health hazards of using water contaminated with *Acanthamoeba* spp. for domestic purposes.

Although there have been no known reports of contracting fatal *Acanthamoeba* infection through groundwater ingestion, there is an increasing number of AK cases reported recently in different parts of the world (Alver *et al.* 2020; Bagga *et al.* 2020; Satitpitakul *et al.* 2021). Also, there was a peculiar demonstration of *Acanthamoeba* spp. in urine samples of critically ill patients (Santos *et al.* 2009). The capacity of *Acanthamoeba* spp. to trigger an immune response in the gastrointestinal tract (GIT) is an argument that is yet to be resolved. Although this is the case, it is important to consider that FLAs have been isolated from the animal intestines and bat guano (Mulec *et al.* 2016; Milanez *et al.* 2017). More importantly, *Acanthamoeba* spp. is known to harbor a plethora of microorganisms and safely host them within the cytoplasm of cystic stages (Scheid 2018). This mechanism enables the latter to evade the immune responses of the host, allowing the process of disease causation. Thus, the capacity of FLAs as Trojan horses to introduce human pathogenic endocytobionts in the GIT and other anatomic sites requires consideration.

In our study, we have identified two potentially pathogenic *Acanthamoeba* spp. isolates, namely IN2.1 (OP411026) and IN2.2 (OP411028) confirmed through an in vitro thermo-tolerance assay. This suggests that groundwater contaminated

Figure 4 | Groundwater sources from sampling sites with positive *Acanthamoeba* spp. growth: (a) Barangay Inhobol collection site 1, (b) Barangay Inhobol collection site 2, and (c) Barangay Sto. Rosario collection site. The collection site shows an unhygienic environment and manual pumps directly in contact with soil.

with *Acanthamoeba* spp. may cause FLA-related infections in humans. This is the first report of *A. astronyxis* in the Philippines. However, its pathogenic potential remains elusive to the present date (Callicott 1968; Martinez 1980).

The findings of several *Acanthamoeba* spp. in groundwater sources in the country necessitate the revisiting of the local national water standards to include the screening of pathogenic protozoans from freshwater sources for human use. Also, the Philippines, at present, has no case definition for FLA-related infections (Milanez *et al.* 2022). This hints at the lack of reports of FLA-related infections in the country as such cases may either be misdiagnosed, underreported, or neglected.

CONCLUSION

This is the first report of *A. astronyxis* genotype T7 in the Philippines. The thermo-tolerance characteristics exhibited by local *Acanthamoeba* spp. from groundwater sources is evidence of a pathogenic potential for humans. To date, the detection of pathogenic protozoans in freshwater sources for human use and a case definition for FLA-related infections is non-existent in the country. It is necessary to craft country policies that address FLA-related health concerns. The role of *Acanthamoeba* to trigger an immune response in the gastrointestinal tract and other anatomic sites is still unclear but requires consideration as it can introduce pathogenic endocytobionts.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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