Detection of *Acanthamoeba* spp. in two major water reservoirs in the Philippines

Giovanni Milanez, Frederick Masangkay, Frieda Hapan, Thea Bencito, Marcus Lopez, Jeffrey Soriano, Abdee Ascaño, Louella Lizarondo, Joseph Santiago, Voravuth Somsak, Manas Kotepui, Amalia Tsiami, Jitbanjong Tangpong and Panagiotis Karanis

ABSTRACT

Water reservoirs are important manmade structures providing water security to deliver clean and safe water for drinking and other purposes to the community. Eighty water samples were collected from Magat and Ipo water reservoirs using purposive sampling between November 2018 and January 2019. Water samples were collected in sterile containers for testing. The samples were cultured in non-nutrient agar and lawned with *Escherichia coli* and incubated at 33 °C. Twelve out of the 80 (15%) water samples were positive for amoebic growth. Light and scanning electron microscopy (SEM) revealed double-walled cystic stages and were initially identified as *Acanthamoeba* spp. based on morphological characteristic in reference to Page's established criteria. Their extracted DNAs were used in polymerase chain reaction using JDP1 and JDP2 primers and confirmed the presence of *Acanthamoeba* DNA in agarose gel electrophoresis. Aligned sequences from PCR products were deposited in GenBank under accession numbers MK886460, MK909919, MK905437, MK910997, MK911021 and MK886514. The presence of potentially pathogenic *Acanthamoeba* spp. in water reservoirs is considered a potential risk for public health, requiring appropriate processing of water in treatment plants.

Key words | *Acanthamoeba* spp., free living amoeba, Ipo reservoir, Magat reservoir, PCR, Philippines

Giovanni Milanez
Frederick Masangkay
Voravuth Somsak
Manas Kotepui
Jitbanjong Tangpong (corresponding author)
Biomedical Science Program, School of Allied Health Sciences,
Walailak University,
Nakhon Si Thammarat 8016, Thailand
E-mail: *rjitbanj@wu.ac.th*

Frieda Hapan
Thea Bencito
Marcus Lopez
Jeffrey Soriano
Abdee Ascaño
Louella Lizarondo
Joseph Santiago
Department of Medical Technology, Faculty of Pharmacy,
Royal and Pontifical University of Santo Tomas,
Manila, Philippines

Giovanni Milanez
Frederick Masangkay
Department of Medical Technology,
Far Eastern University,
Manila, Philippines

Amalia Tsiami
London Geller College of Hospitality and Tourism,
University of West London,
London, UK

Panagiotis Karanis
University of Nicosia Medical School,
Anatomy Centre, Nicosia, Cyprus

INTRODUCTION

Water reservoirs are important manmade structures providing water security to deliver clean and safe water for drinking and other purposes to the community (Đorđević & Dasic 2011). The World Health Organization (WHO) estimates that around 3.4 million people die every year due to water-related diseases and it is becoming the leading cause of morbidity and mortality in the world (Berman 2009). The genus *Acanthamoeba* is considered an important pathogen of our time due to its ability to cause opportunistic and non-opportunistic infections in humans (Marciano-Cabral & Cabral 2003). These eukaryotic organisms belong to a group of amoebae known as free living amoebae (FLA) that are

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widely distributed in the environment as well as in recreational pools, cooling towers, water tanks, tap water systems, dental units, air conditioning equipment and others (Scheid 2018; Di Filippo *et al.* 2015). Furthermore, FLA have been reported to be isolated from bat guano (Mulec *et al.* 2016) and in bromeliads (Landell *et al.* 2013). *Acanthamoeba* spp. was detected in Brazil's recreational waters, where three genotypes were identified (Caumo & Rott 2011). *Acanthamoeba* spp. has been isolated in Asia and the Pacific region water resources including thermal hot springs in Taiwan (Kao *et al.* 2012) and Thailand (Lekkla *et al.* 2005), as well as water reservoirs in China (Lass *et al.* 2017) and Japan (Edagawa *et al.* 2009). There are currently 18 species of *Acanthamoeba* and these are classified into three groups based on the features of endocyst and ectocyst (Ubelaker 2014). *Acanthamoeba* spp. are known to cause two major human infections: granulomatous amoebic encephalitis (GAE) which affects the central nervous system and usually is fatal to the host and *Acanthamoeba* keratitis (AK) that can cause blindness (Khan 2006). Moreover, *Acanthamoeba* spp. are reported to cause cutaneous infections, especially in immunocompromised patients, which are described as erythematous papules, pustules or linear lesions with erythematous background (Deluoi *et al.* 1996; Casper *et al.* 1999). Aside from its virulence to cause infection, *Acanthamoeba* spp., being a natural phagocyte, is considered a 'Trojan horse' which is able to harbour endosymbionts such as viruses, yeasts, protists and bacteria that are pathogenic to humans (Greub & Raoult 2004).

Isolation and characterization studies of *Acanthamoeba* spp. in the Philippines are limited to clinical samples from contact lenses (Rivera & Adao 2009; Buerano *et al.* 2014) and nasal swabs (Cruz & Rivera 2014). Point source isolation and identification of *Acanthamoeba* from main water reservoirs have yet to be studied in the country. Magat and Ipo water reservoirs are considered as the main water reservoirs supplying potable water in the island of Luzon, Philippines. The Magat reservoir contributes to water irrigation for crops, fish production, domestic uses and electricity generation (Elazegui & Combalicer 2004) while the Ipo reservoir, which is linked to the Angat-Umiray-Ipo watersheds, provides 98% of the water needs of the capital Metro Manila (WWF). Being the main sources of water for the public and considering the frequency of human contact, the need to further explore the presence of potential pathogenic FLAs in these environments is crucial to provide awareness and measures that can be taken to prevent human infections. In this study, we isolate and identify possible FLAs present in the two water reservoirs.

METHODS

Water sample collection, processing and culture

A total of 80 water samples were taken from Magat and Ipo water reservoirs (40 water samples in Magat; 40 water samples in Ipo) using purposive sampling between November 2018 and January 2019. Four sampling sites in Ipo watershed were identified, namely, site one – Sitio Bininit (14°53′53″N 121°10′16″E), site two – Sitio Paco (14°53′16″N 121°9′37″E), site three – Sitio Sapang Munti (14°52′13″N 121°9′8″E), site four – Sitio Bitbit (14°54′1″N 121°8′30″E), while the four sampling sites for Magat watersheds were site one – Baligatan (16°48′45.9″N 121°26′47.9″E), site two – Isla Verde (16°48′43.5″N 121°26′34.7″E), site three – Isla Verde (16°48′56.9″N 121°26′38.8″E) and site four – Callao (16°49′09.6″N 121°26′36.3″E). Sample sites were selected based on accessibility, presence of community near the shore as proof of anthroponotic activity or due to the presence of fish farms. Two hundred and fifty mL of water samples were then collected at approximately 10–20 cm depth from the surface and placed in sterile containers (Milanez *et al.* 2019). The samples were transported to the laboratory at the Department of Medical Technology, Faculty of Pharmacy, University of Santo Tomas, Philippines to identify amoebic growth using culture methods. Samples were transferred to Falcon tubes and pelleted at 3,000 rpm for 15 min. The resulting pellets were evenly lawned to non-nutrient agar (NNA) lawned with *Escherichia coli* and were incubated at 33 °C (Schuster 2002).

Light and scanning electron microscopic (SEM) analysis of NNA plates

NNA lawned with *E. coli* culture plates were put in an incubator set at 33 °C and were examined daily for amoebic growth using a regular compound microscope

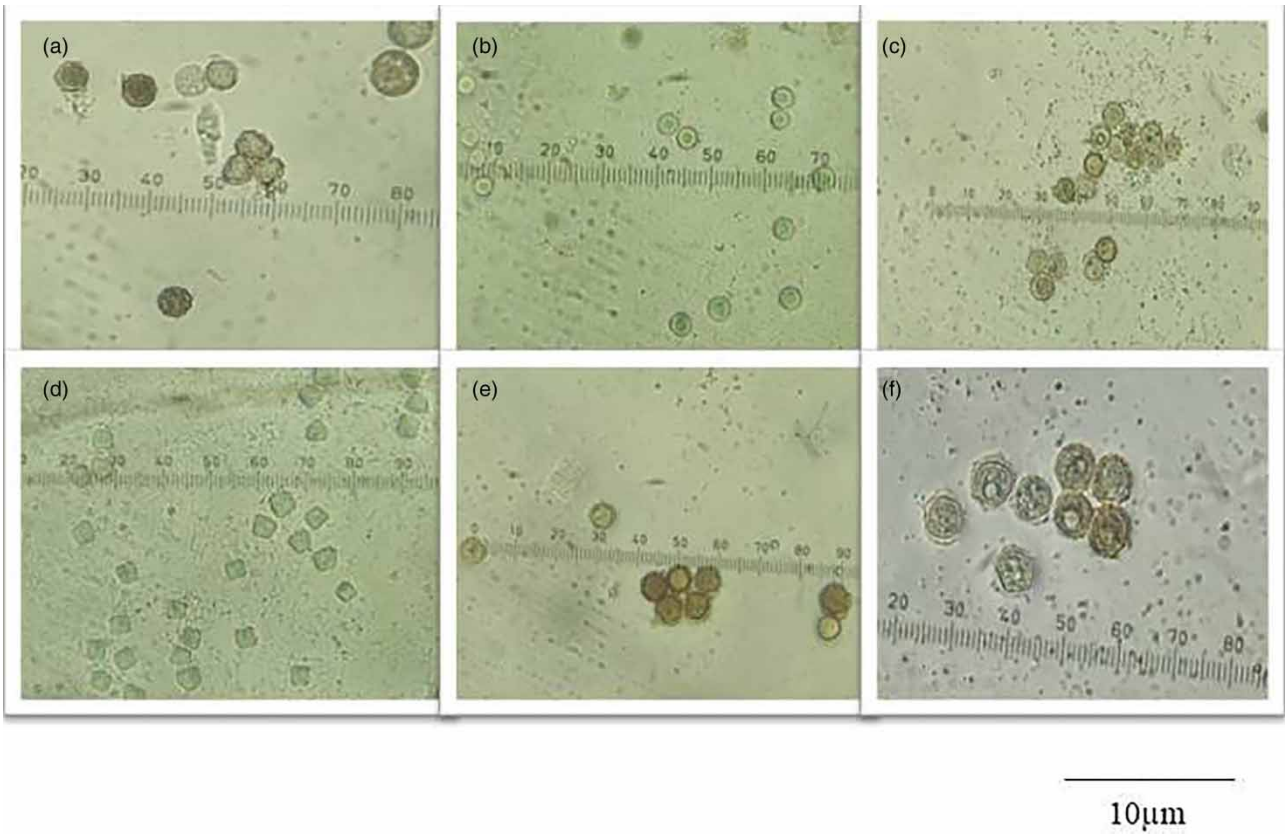

Figure 1 | Micrograph of *Acanthamoebae* spp. cysts in Magat Dam showing characteristic double-walled cysts. Cyst sizes are approximately 6–10 µm. (a) MS4-5, (b) MS4-2, (c) MS3-4, (d) MB4-3, (e) MB3-2, (f) MB1-5. Scale is set as 10 µm.

(Nikon Eclipse E100) for 14 consecutive days before being declared negative as per the protocol established by Page (1988). Plates that showed cystic forms and motile trophozoites were further tested and sub-cultured (Milanez *et al.* 2019). This procedure was done until a homogenous culture of FLA was obtained. Electron microscopy was done using TM300 Hitachi tabletop SEM. Briefly, the surfaces of positive NNA sub-cultured plates were gently scraped to detach cysts and trophozoites and placed onto coverslips. Coverslips were then attached to carbon tape secured on the metal SEM platform for further analysis.

DNA extraction and molecular analysis

Culture plates with positive trophozoites and cysts as observed by the microscopic analysis were transported to the Department of Medical Technology, Far Eastern University Manila, Philippines for further molecular analysis. Trophozoites and cysts were harvested by flooding the surface of the agar with cold phosphate-buffered saline solution and by gently scraping the agar surface with a sterile scalpel blade and then aspirated (Milanez *et al.* 2017). Aspirated fluid was then transferred to microcentrifuge tubes and DNA was extracted using Macherey-Nagel DNA extraction kit (NucleoSpin®) following the manufacturer's protocol. DNA was made to react to polymerase chain reaction using primer set JDP1 5′GGCCCAGATCGTTTACCGTGAA-3′ and JDP2 5′TCTCACAAGCTGCTAGGGAGTCA-3′. PCR conditions were set as follows: 95 °C for 7 min initial denaturation, 40 cycles of denaturation at 95 °C for 1 min, 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.* 2004).

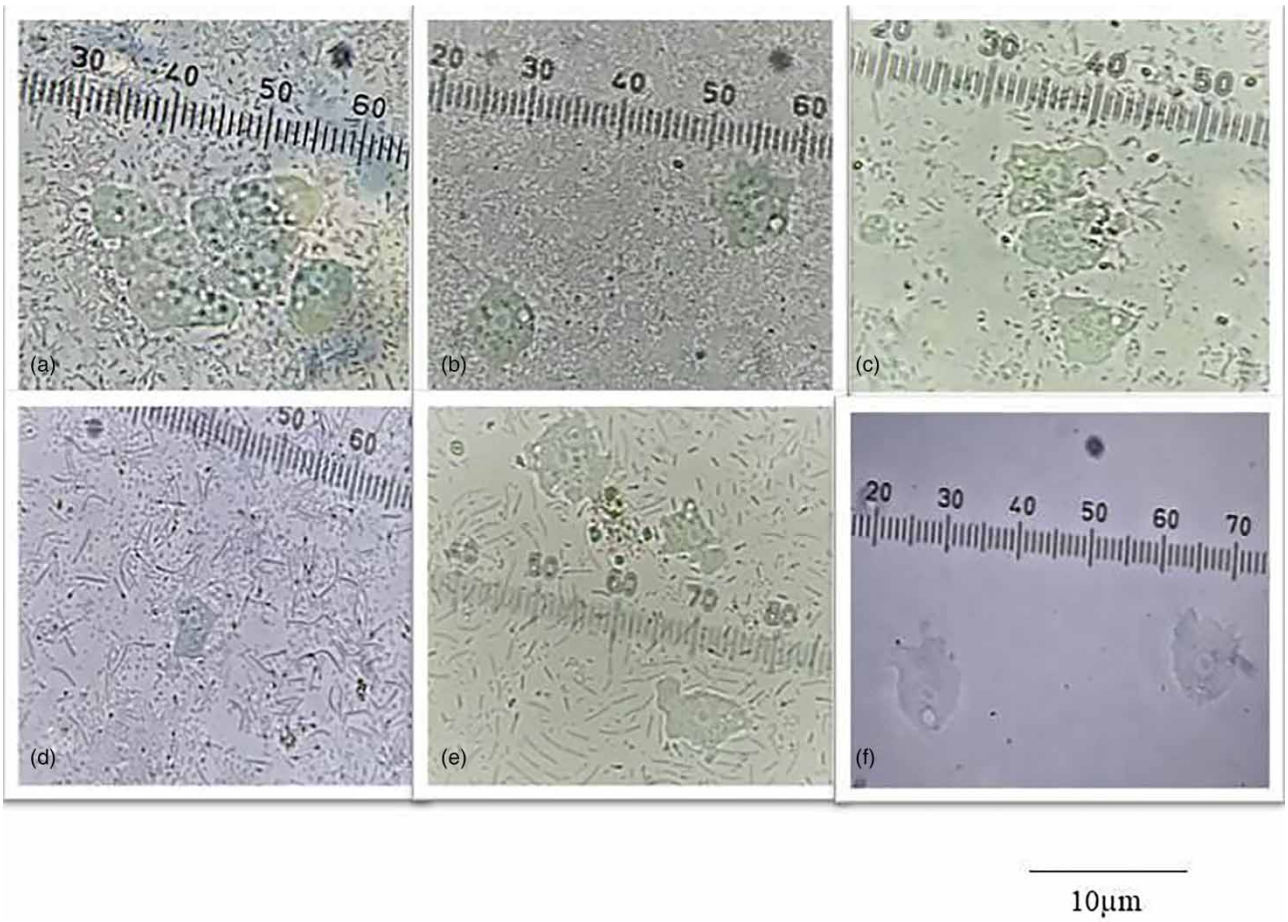

Figure 2 | Micrographs of isolated trophozoites in Ipo watershed showing single pseudopodia and distinct nucleus. Trophozoites exhibit multiple vacuoles and sluggish progressive movement. (a) IS1 B2, (b) IS1 B3, (c) IS1 B5, (d) IS2 B3, (e) IS3 B2, (f) IS1 B3.

DNA sequencing and phylogenetic analysis

To further identify *Acanthamoeba* genotypes, further sequencing and phylogenetic analyses were performed. In detail, a 1.5% agarose gel stained with ethidium bromide was used to visualize PCR amplicons. Amplicons were sent to a commercial sequencing company (Macrogen, Seoul, South Korea) for further sequencing. Sequences were aligned using ClustalW of BioEdit with careful visual consideration of gaps and ambiguous sequences and deposited with GenBank afterwards. The DNA sequences of isolates IS1B5, IS4B3, MS45, MS42, IS2B1 and IS1B2 obtained from this study were deposited in the GenBank database and are available under accession numbers MK886460, MK909919, MK905437, MK910997, MK911021 and MK886514, respectively.

RESULTS

Light microscopic and scanning electron microscopic (SEM)

A total of 12 out of 80 (15%) water samples were positive for amoebic growth obtained from Magat and Ipo watersheds. Specifically, positive growths of FLA were observed from samples in sites one, three and four in Magat reservoir and sites one, two and three of Ipo watersheds. Light microscopic images of both cysts and trophozoites are presented in Figures 1 and 2, respectively. Cystic stages seen under light microscopy are described as irregularly shaped with distinct double wall measuring approximately 10 μm. Trophozoites observed microscopically have a sluggish directional movement with single pseudopodia.

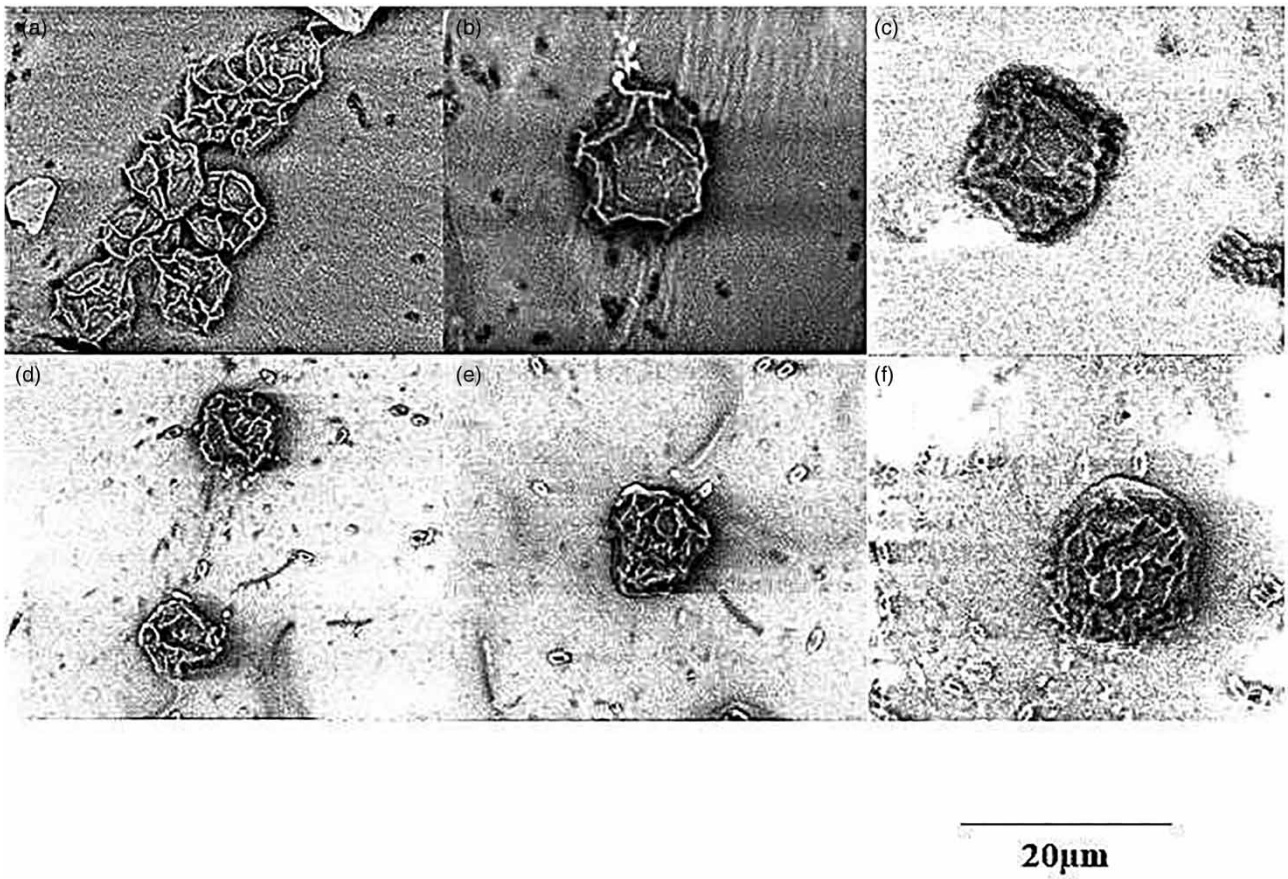

Figure 3 | Scanning electron micrographs of isolated cystic stages in Magat and Ipo water reservoirs showing characteristic wrinkled appearance of ectocyst (outer cyst wall) of *Acanthamoebae* spp. (a) and (b) MB4-3 isolate cystic stages, (c) IS2 B1 isolate, (d) and (e) IS1 B3 isolate, (f) IS2 B1 isolate.

The cytoplasm is somewhat granular and some are highly vacuolated. Furthermore, SEM of cystic stages revealed cystic stages with wrinkled surface appearance as shown in Figure 3. All the findings were morphologically compared based on Page's established criteria in respect to form, size and shape (Page 1967) and were initially classified morphologically as *Acanthamoeba* spp. Enflagellation testing of amoeba isolates was not performed further due to limitations of the methods on the reliability of the test to differentiate amoebae from other groups (De Jonckheere *et al.* 2001).

Molecular analysis result

Polymerase chain reaction results using JDP1 and JDP2 primers showed band formation between 400 and 500 bp in agarose gel electrophoresis showing the presence of DNA for isolates IS1B5, IS4B3, MS45, MS42, IS2B1 and IS1B2. Isolate M5I3 belonging to *Acanthamoeba* genotype T5 was used as a positive control. Further sequencing of PCR amplicons and phylogenetic analysis was done and confirmed the presence of *Acanthamoeba hatchetti* belonging to genotype T11, *Acanthamoeba* spp. belonging

Table 1 | Isolated *Acanthamoebae* spp. in Magat and Ipo water reservoirs with assigned accession numbers from GenBank

Isolate	Genotype	GenBank Accession Number	Location
IS1B5	T3	MK886460	Ipo water reservoir
IS4B3	T3	MK909919	Ipo water reservoir
IS2B1	T3	MK911021	Ipo water reservoir
IS1B2	T4	MK886514	Ipo water reservoir
MS45	T11	MK905437	Magat reservoir
MS42	T5	MK910997	Magat reservoir

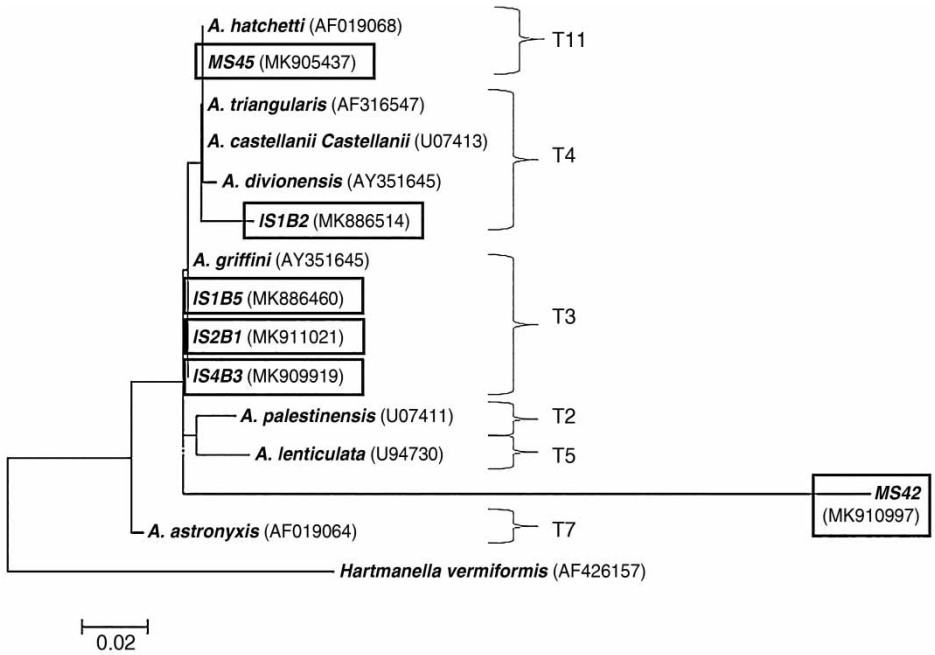

Figure 4 | Maximum likelihood tree of *Acanthamoeba* spp. isolates (in boxes). Tree was constructed using Tamura 3 parameter using MEGA 7 application. Phylogenetic analysis was carried out using 1,000 bootstrap replicates.

to genotype T3, *Acanthamoeba* spp. belonging to genotype T4 in Magat and Ipo reservoirs. Table 1 shows the different isolated *Acanthamoeba* spp. in both Magat and Ipo water reservoirs. Maximum likelihood tree using Tamura 3 parameter model was constructed as suggested as the best tree model using MEGA 7 application (Figure 4). Table 2 shows the reference strains used to construct the tree.

DISCUSSION

The Magat and Ipo water reservoirs are two important man-made water reservoirs in the Philippines. Magat water reservoir supplies hydroelectric energy, aquatic produce, irrigation and a potable water source to three provinces, namely, Nueva Vizcaya, Quirino and Isabela (Baleta & Bolaños 2016) while Ipo water reservoir is a part of a

Table 2 | Reference strains of *Acanthamoeba* spp. used for phylogenetic construction in the present study

Organism	GenBank Accession Number	Source/Location	References
Acanthamoeba hatchetti	AF019068	Corneal scrapings	Ledee *et al.* (1996)
Acanthamoeba triangularis	AF316547	Human feces	Chung *et al.* (2005)
Acanthamoeba castellanii	U07413	Yeast culture	Gast *et al.* (1996)
Acanthamoeba divionensis	AY351646	Soil	Chung & Kong (2003)
Acanthamoeba griffini	U07412	No source given	Gast *et al.* (1996)
Acanthamoeba palestinensis	U07411	Yeast culture	Gast *et al.* (1996)
Acanthamoeba lenticulata	U94730	No source given	Schroeder-Diedrich *et al.* (1998)
Acanthamoeba astronyxis	AF019064	No source given	Schroeder-Diedrich *et al.* (1998)
Hartmanella vermiformis	AF426157	No source given	Walochnik *et al.* (2002)

larger system called the Angat–Ipo–La Mesa water system that directly supplies water to treatment facilities which are then supplied directly to Metro Manila (Masangkay *et al.* 2014). This study has confirmed the presence and the biodiversity of *Acanthamoeba* spp. in two major water reservoirs in the Republic of the Philippines, namely, Magat and Ipo water reservoirs, and to the best of our knowledge, is the first report in the country. The presence of FLAs, specifically *Acanthamoeba* spp., in these two major water reservoirs, is a clear indication of the high risk that these FLAs can be incorporated into the water being supplied to water treatment facilities and in return to households as tap water, increasing the risk of potential transmission to humans.

The ability of *Acanthamoeba* spp. to graze with a number of potentially pathogenic bacteria such as *Legionella* and *Mycobacteria* (Greub & Raoult 2004) and to safely co-exist as endosymbionts within them suggests that there is a possibility that these microorganisms may escape disinfection during the treatment process (Berry *et al.* 2010). More importantly, the two water reservoirs directly supply tap water to a very large population in the country, thus, the presence of these FLAs in the water may be considered as a public health issue. The presence of genotypes T3, T4 and T11 in the two water reservoirs suggests a high possibility of transmitting *Acanthamoeba* keratitis (AK) to people who use tap water from faucets to wash their faces, considering that these genotypes are most often associated with AK, as suggested by some studies (Ledee *et al.* 1996; Booton *et al.* 2005). This has been proven to be true with a relatively rare case of AK involving a non-contact lens wearer in the Philippines with a history of using tap water to wash his face before acquiring the condition (Buerano *et al.* 2014).

Finally, genotypes T3 and T4 are considered the most common genotypes isolated in the environment due to the high resistance to chlorine of their cystic stages whereas genotype T11 is considered susceptible (Shoff *et al.* 2008). The isolation of *Acanthamoeba* genotype T11 in Magat water reservoir suggests low levels of chlorination as do reports from drinking waters of hospitals in Iran (Bagheri *et al.* 2010), although the exact measurement of chlorine was not carried out in this study. Chlorination is considered as an initial step in water disinfection due to its ability to inactivate a number of pathogenic microorganisms (Environmental Protection Agency 2011).

This study has shown the importance of point source isolation of potential pathogenic organisms such as *Acanthamoeba* spp. Further, images obtained from SEM analysis have provided clear differences of cyst size and morphology of the four different genotypes isolated from two water reservoirs in the Philippines. The detection of potentially pathogenic organisms in the environment, particularly in water reservoirs, in which human activities such as swimming and use of water for other purposes like bathing are likely to occur, will further provide awareness to the community of their presence, thus avoiding severe or even fatal health conditions.

CONCLUSIONS

The results of this study produce the first description through scanning electron microscopy and molecular techniques of *Acanthamoeba* isolates in water reservoirs in the Philippines. The presence of *Acanthamoeba* spp. that can cause conditions such as GAE and AK in water reservoirs increases the chances of human contact, since water coming from these environmental structures is directly provided as water sources for different human activities, which include bathing, washing and, to some extent, use as potable water. Not only is their the chance of acquiring FLAs, but also the infection of potential pathogenic bacteria that act as endosymbionts.

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