Occurrence and the first report of *Naegleria australiensis* presence in a major lake in the Philippines

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ABSTRACT

Laguna de Bay or Laguna Lake is one of the six major lakes in the Philippines to be in close contact with population activities due to the expansion of urban settlements in the immediate cities surrounding the lake, thus pushing the population to settle upon its shores. To date, there are no data showing the biodiversity of free-living amoebae (FLA) present in this lake. The present study aims to isolate and identify the FLA present in Laguna de Bay, Philippines. Thirty subsurface water samples were taken from Laguna De Bay using random purposive sampling in May 2018 and were examined for amoebic growth under light microscopy (LM). Results show that 8 out of 30 (26.6%) water samples were positive for amoebic growth and were further tested for more advanced data and genetic variation of the species. Initial molecular analysis using polymerase chain reaction (PCR) and sequencing showed the presence of potentially pathogenic FLA *Naegleria australiensis* (MK418954). The detection of potential pathogenic FLA in lakes and dams may prove useful in preventing and controlling possible human infections in the country. More data from this study will aid in public awareness and establishing safety guidelines and control programs.

Key words | free living amoebae, *Naegleria australiensis*, PCR, Philippines, sequencing, surface water

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INTRODUCTION

The Philippines is an archipelago blessed with abundant natural resources. Among these are natural freshwater lakes that are being used by the population for both recreation and livelihood purposes. Although freshwater lakes are of significant importance to the community, the study of natural lakes in the Philippines is still considered fragmented and inconsistent, and mainly focused on marine biodiversity studies, particularly fish (Papa & Briones 2017). For this reason, studies involving the freshwater ecosystem have yet to be explored and considered as a niche to several scientific studies concerning the biodiversity of different organisms aside from fish.

Free-living amoebae (FLA) are one of the many organisms that thrive in freshwater systems and are considered ubiquitous in the environment (Scheikl *et al.* 2014). The World Health Organization (WHO) has named four genera of FLA that can cause fatal central nervous system infections to humans, being *Naegleria* spp., *Acanthamoeba* spp., *Balamuthia* spp., and *Sappinia* spp. (World Health Organization 2003). Among the four FLAs mentioned, *Naegleria* spp. accounts for the highest number of deaths due to primary amoebic meningoencephalitis (PAM). PAM is characterized as severe, fulminant encephalitis that is fatal in 5–7 days without medical intervention (Visvesvara *et al.* 2007). The rapid progression of the disease prevents the body from reacting immunologically resulting in the death of the host. Although a number of therapeutic drugs have been

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developed to eliminate the parasite from the body, the host response is still considered as a crucial factor for survival. This is true in some reported cases where the host was given the same therapeutic drug but resulted in different outcomes (Gupta *et al.* 2009; Saleem *et al.* 2009; Shakoor *et al.* 2011; Movahedi *et al.* 2012). Due to its rapid onset as well as the difficulty in diagnosis and treatment management, PAM has become a major public health concern to clinicians and researchers. A history of having contact with fresh water proves to be valuable information in the early diagnosis of PAM and may be instrumental in providing an effective therapeutic regimen. Unfortunately, medical protocols tend to overlook this piece of information, especially when general symptoms present that most often would lead clinicians to suspect bacterial or viral meningitis instead of the protozoan form as seen in recent cases (Johnson *et al.* 2012; Kemble *et al.* 2012; Cope *et al.* 2017). In the Philippines, there are no reported cases of PAM despite *Naegleria fowleri*'s isolation in Lake Taal and soil samples (Fontanilla *et al.* 2001), as well as pathogenicity testing of Philippine isolates in mice models (Simeon *et al.* 1990) having been reported.

Laguna de Bay or Laguna Lake is one of the six major lakes in the Philippines in close contact with the population due to the expansion of urban settlements in the immediate cities surrounding the lake, thus pushing the population to settle upon its shores (Guzman & Estiva 2006). The lake is considered a primary source of freshwater fish such as *Chanos chanos* and *Oreochromis niloticus* that are consumed by people living in Manila as well as the neighboring towns and cities. Current biodiversity studies being conducted in the lake include those of fish, zooplankton, phytoplankton, as well as tests for heavy metal, alkalinity, and fungal fish diseases (Tamayo-Zafaralla *et al.* 2002). Currently, only studies that involve parasitic crustaceans that affect fish (Lopez 2001) are available in the literature and there is a need to conduct studies of parasitic organisms that can impact the local population that works and lives on the shores of the Laguna de Bay. Here, we describe investigations on the occurrence, isolation, and identification of FLA present in Laguna De Bay, the Philippines.

METHODS

Laguna De Bay

The lake is considered as the largest freshwater body in the Philippines with a total surface area of 900 km^2, a depth of 2.5 m and an elevation of 1 m above sea level. The lake is bordered by three provinces, namely, Metro Manila in the northwest, the province of Rizal in the north and northeast, and the province of Laguna in the east, west, and southwest. The lake is drained by small streams and the Pasig River which is its outlet to Manila Bay. The water of the lake is relatively warm, possibly due to volcanic hot springs coming from the towns of the province of Laguna located on the southern part of the lake. The Laguna Lake region is considered as one of the most important and dynamic freshwater resources in the Philippines. It contributes to the growing economy of the country in terms of its uses such as a source of aquaculture, agriculture support, and an alternative water source for the community surrounding the lake. The lake is thus considered as a 'resource shed' of the major cities surrounding the lake, in particular, the capital, Manila (Guzman & Estiva 2006). This translates to the lake's importance in providing economic and environmental support to the community.

Sampling sites

Eight sites around the lake were selected as water collection points for this study. All sampling sites are described as having communities living near the shore or having direct access to the lake except for Site 1 in Barangay Poblacion where the shore is accessible through a privately owned duck farm. All sampling sites have makeshift fishing pens which indicate that fishing is considered as the way of life of people in the area.

Sample collection and processing

Thirty subsurface water samples were taken from Laguna de Bay using random purposive sampling in May 2018. A total of eight sites surrounding the lake were selected as collection points of water for sampling. These sites were: Site 1,

Barangay Poblacion (14°30′38.8″N 121°08′38.9″E); Site 2, Sitio Kuhala (14°30′20.4″N 121°04′21.3″E); Site 3, Sitio Wawa (14°20′19.6″N 121°28′30.4″E); Site 4, Sitio Masili (14°11′03.2″N 121°12′04.6″E); Site 5, Sitio Malanggam (14°21′47.6″N 121°14′20.3″E); Site 6, Sitio Tuna (14°19′53.5″N 121°14′39.9″E); Site 7, Sitio Kabagatan (14°19′10.2″N 121°13′26.0″E); and Site 8, Sitio Kinaboogan (14°22′36.1″N 121°13′12.7″E). Sample sites were selected based on accessibility, presence of community near the shore as proof of anthroponotic activity, or by the presence of fish farms. 250 mL of water samples were collected at approximately 10 to 20 cm from the surface and placed in sterile containers. The samples were transported to the laboratory at the Department of Medical Technology, Far Eastern University, Manila, Philippines for further testing. Samples were transferred to Falcon tubes and pelleted at 3,000 rpm for 15 min. The resulting pellets were evenly lawned on previously prepared non-nutrient agar (NNA) lawned with *Escherichia coli* and were incubated at 35 °C. Briefly, 15 g of NNA was dissolved in 1,000 mL of Page's Amoeba Saline (PAS) solution. The mixture was autoclaved, poured, and solidified in sterile disposable Petri dishes. The *E. coli* colonies on NNA plates were harvested by adding 2 mL of PAS solution and pipetted to detach the *E. coli* colonies from the NNA agar surface. The suspensions were transferred to sterile containers and diluted 2–3 times using PAS solution. Ten drops of the suspension were transferred and spread evenly on the surface of NNA. Plates were used immediately for cultivation or stored at 4 °C up to 3 weeks.

Microscopic analysis

Culture plates were examined daily for amoebic growth using a regular compound microscope (Nikon Eclipse E100) for 14 consecutive days before being declared negative. Plates that showed cystic forms and motile trophozoites were further tested and subcultured. Briefly, agar surface of positive plates was observed under light microscopy (Nikon Eclipse E100) to identify the best spot of FLA growth, after which a spot of approximately 1 × 1 cm was cut using a sterile scalpel blade. The agar block was placed upside down onto a new NNA lawned *E. coli* plate. The plate was put in an incubator set at 35 °C. This procedure was done until an axenic culture was obtained.

DNA extraction and 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. Fluid was then transferred to microcentrifuge tubes and DNA was extracted using Macherey-Nagel DNA extraction kit (NucleoSpin®) following the manufacturer's protocol. DNAs were made to react to polymerase chain reaction (PCR) using 18S rRNA universal primers EukA 5′-AACCTGGTTGATCCTGCC AGT-3 and EukB 5′-TGATCCTTCTGCAGGTTCACCTAC-3 (Medlin *et al.* 1988). PCR conditions were set as follows: 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 (Milanez *et al.* 2017).

DNA sequencing and phylogenetic analysis

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 in GenBank afterwards. A maximum likelihood (ML) tree was constructed using MEGA7 application which is based on the best model. Bootstrap resampling was carried out using 1,000 replicates. The DNA sequence of isolate LB2 obtained from this study was deposited in the GenBank database and is available under accession number MK418954.

RESULTS

Microscopic results

A total of eight out of 30 (26.6%) water samples were positive for amoebic growth and came from two collection sites, Site 2 (Sitio Kuhala) and Site 3 (Sitio Wawa). Cystic stages seen under light microscopy are described as circular with a distinct central nucleus measuring approximately

10 μm. The trophozoite that was observed in the positive culture, isolate LB2 (MK418954), is described as being monopodial with a sluggish directional movement. The cytoplasm is somewhat granular and single pseudopodia are observed. All the findings were morphologically compared based on Page's established criteria in respect to form, size, and shape (Page 1967) and initially classified morphologically as *Naegleria* spp. Enflagellation testing of amoeba isolates was not further performed due to some speculation regarding the reliability of the test to differentiate some amoebae from other groups (De Jonckheere *et al.* 2001).

Molecular results

DNA from the isolates were made to react to polymerase chain reaction using universal primers Euk A and Euk B targeting the 18S region (Medlin *et al.* 1988). Further sequencing of PCR amplicons was done and confirmed the presence of *N. australiensis* (MK418954) in Laguna de Bay. Agarose gel electrophoresis results of PCR amplicons showed distinct band formation between 800 and 1,100 bp suggestive of the presence of FLA (Di Filippo *et al.* 2015). Figure 1 represents the ML phylogenetic tree analyses of *N. australiensis* (MK418954) isolate using the Jukes–Cantor model as the best model to use in the construction of the tree using MEGA 7 application. Table 1 shows the reference strains from GenBank that were used for the construction of the tree.

DISCUSSION

This study confirms, through molecular testing, the presence of potentially pathogenic FLA *N. australiensis* (MK418954) in Laguna de Bay and, to the best of our knowledge, the first report of its presence in this lake. Phylogenetic analysis has shown that isolate LB2 (MK418954) has a close resemblance in sequence to the reference strain of *N. australiensis* (AB128053) isolated from tap water in Japan (Edagawa *et al.* 2009). *N. australiensis* was confirmed a pathogenic FLA when pathogenicity testing was performed in experimental mice in which important differences of the two species in terms of virulence and course of infection were noted. The first was the level of virulence where *N. fowleri* demonstrated more virulence compared with *N. australiensis*. Second is that the survival

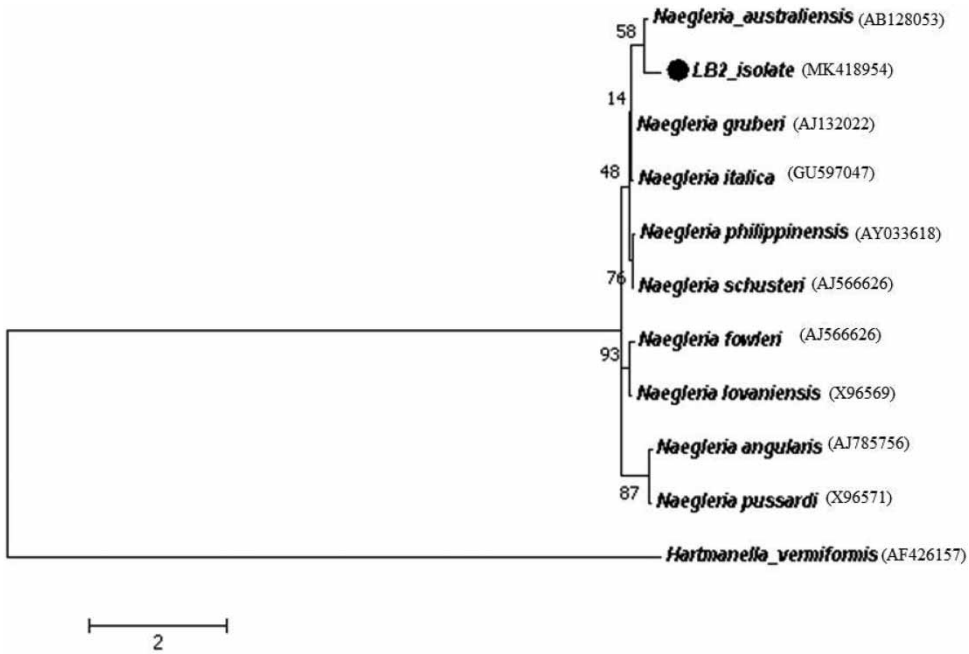

Figure 1 | ML phylogenetic relationships of the partial 18S rRNA sequence of isolate LB2 from this study and reference strains of *Naegleria* spp. sequences deposited with GenBank. Accession numbers are in parentheses. Bootstrap value is set at 1,000 replicates. The tree was constructed using the Jukes–Cantor model using MEGA7.

Table 1 | Reference strains of *Naegleria* spp. used for phylogenetic construction in the present study

Organism	GenBank accession number	Source/Location	References
Naegleria italica	GU597047	Brackish water/Taiwan	Hsu *et al.* (submitted)
Naegleria gruberi	AJ132022	Water station/France	Pelandakis *et al.* (2000)
Naegleria schusteri	AJ566626	Environmental/Belgium	De Jonckheere (2004)
Naegleria philippinensis	AY033618	Fresh water/Philippines	Fontanilla *et al.* (2001)
Naegleria australiensis	AB128053	Tap water/Japan	Edagawa *et al.* (2009)
Naegleria fowleri	AJ132027	Water station/France	Pelandakis *et al.* (2000)
Naegleria lovaniensis	X96569	Environmental/Belgium	De Jonckheere (1998)
Naegleria pussardi	X96571	Environmental/Belgium	De Jonckheere (1998)
Naegleria angularis	AJ785756	Environmental/Belgium	De Jonckheere & Brown (2005)
Hartmanella vermiformis	AF426157	No source given	Walochnik *et al.* (2002)

time for *N. australiensis* is much longer compared with *N. australiensis* infection and both showed similarity in histopathologic changes as with *N. fowleri* infection (De Jonckheere *et al.* 1983). Although there is no further evidence whether the same isolate in this study would provide the same pathogenicity results since testing in mice models was not performed due to time and resource constraints, we believe that further testing regarding this matter will support its confirmation. Laguna de Bay is considered as the largest lake in the Philippines and a vital source of livelihood such as fishing, crop irrigation, and hydroelectric power to communities living around its shore (Palma *et al.* 2002). In a 2013 government report, the lake was divided into four sections based on water quality (presence/absence of nitrates, phosphates, chlorophyll, dissolved oxygen, biochemical oxygen demand, and total coliforms) and presence or absence of aquaculture. These sections are West Bay, Central Bay, East Bay, and South Bay (LLDA 2013). The report concluded that the section that scored the highest in water quality is the East Bay while the Central Bay scored the lowest. In terms of aquaculture, the West Bay has the highest number of commercial fish pens as well as the most heavily populated section of the lake. Site 2 (Sitio Kuhala) and Site 3 (Sitio Wawa), where *N. australiensis* (MK418954) was isolated, are located in the East and West Bay sections of the lake. Central and South Bay proved to be negative. In this study, we offer two factors that might have contributed to the presence of this FLA in the area compared with the sections of the lake where its presence was not detected. One factor could be the presence of aquaculture, which is predominant in the East and West Bay sections of the lake. As mentioned previously, the West Bay area has a higher percentage of commercial fish farms in comparison with other parts of the lake. The presence of abundant fish farms in these sections may have influenced the presence of these FLAs, as suggested by some studies on the ability of FLA to use fish as potential biological reservoir hosts (Dyková *et al.* 2001; Milanez *et al.* 2017).

Another factor is the current condition of the water quality of the lake. The lake's water is considered as significantly stressed due to domestic and industrial waste coming from people and industrial plants around it (Guerrero 1999). It is possible that thermal pollution may have influenced the growth of FLA in the West Bay area since this section of the lake is immediately located nearby industrial plants and factories, similar to a case in Belgium (De Jonckheere *et al.* 1975). This may have caused the water temperature in that area to be optimum for FLA to grow. It should be stressed that the water temperature at the time of collection in Site 2 and Site 3 was 36 °C and 37 °C, respectively, both higher compared to the rest of the sampling sites (below 35 °C). The absence of nearby industrial plants in the Central and South Bay areas may have been the reason for the difference in temperature and since mainly these sites are agricultural. The East Bay moreover, has been reported to have high levels of heavy metals, which are: Zn, Cr, As, Cu, Pb, Cd, and Hg (Tamayo-Zafaralla *et al.* 2002). The levels of these heavy metals were said to be greater than the required safe levels for aquatic life to flourish. This is true, most especially

of the West Bay section of the lake which was identified as being industrialized due to the number of industrial plants located in this part of the lake (Tamayo-Zafaralla *et al.* 2002). Despite this, it was possible to isolate FLA in such water conditions. This suggests that FLA, such as *N. australiensis* (MK418954) isolate, are able to adapt to such harsher environments or possibly the presence of Hg-resistant bacteria that may be found in water may have served as a food source for this FLA, as suggested by some studies done *in vitro* (Hagneré & Harf 1993). Whatever the reason for its ability to survive in such an environment, the remarks stated previously should be further investigated.

In a 2009 report of the water quality status of the lake, the West and East Bay sections of the lake were classified as class A status which makes them fit for water supply and contact recreational swimming (Manda *et al.* 2009). The presence of a potentially pathogenic FLA may impose public health concerns due to increased chances of human contact with infected waters through swimming. Although contact activities such as swimming are unavoidable, especially during the summer months, special care should be taken during swimming and spear fishing that are practiced in some parts of the lake.

CONCLUSION

Based on the results obtained, this study produces the first report of a potential pathogenic FLA *N. australiensis* (MK418954) in Laguna de Bay, Philippines. The results indicated the ability of this FLA to continue to thrive in adverse water conditions. The presence of this FLA in the waters of a major lake which is considered a vital source of food, potable water source, and a potential recreational site may pose a public health threat by transmission through contact activities such as swimming. Further testing of isolates through possible pathogenicity spectra in different mammalian and non-mammalian hosts is recommended.

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