Experimental Parasitology 183 (2017) 201–206

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Molecular identification of *Vermamoeba vermiformis* from freshwater fish in lake Taal, Philippines

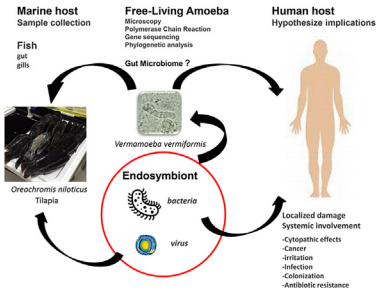
Giovanni D. Milanez [a, *], Frederick R. Masangkay [a], Rey C. Thomas [b], Ma Olive Grace O. Ordona [a], Gabriel Q. Bernales [a], Vyana Camille M. Corpuz [a], Hannah Selina V. Fortes [a], Charezze Margarette S. Garcia [a], Lara Camille Nicolas [a], Veeranoot Nissapatorn [c]

[a] Department of Medical Technology, Far Eastern University, Manila, Philippines
[b] Department of Biology, Far Eastern University, Manila, Philippines
[c] School of Allied Health Sciences (Southeast Asia Water Team), Walailak University, Nakhon Si Thammarat, Thailand

HIGHLIGHTS

- Free-Living Amoebas were isolated from Freshwater fishes from Lake Taal, Philippines.
- Molecular identification and phylogenetic analysis of FLAs and *Vermamoeba* sp. from fresh water fish.
- *Vermamoeba vermiformis* was isolated from the gut of a freshwater fish *Oreochromis niloticus* tilapia.
- *Oreochromis niloticus* tilapia fish gut microbiome.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 20 July 2017
Received in revised form
6 September 2017
Accepted 11 September 2017
Available online 13 September 2017

Keywords:
Free living amoebae
Fresh water fishes
Lake Taal
Phylogeny
Philippines
Vermamoeba vermiformis

ABSTRACT

Free Living Amoebae (FLA) are considered ubiquitous. FLAs may infect various biological organisms which act as reservoir hosts. Infected freshwater fishes can pose a public health concern due to possible human consumption. This study aims to identify possible pathogenic FLAs present in freshwater fishes. Seventy five (75) *Oreochromis niloticus* were studied for the presence of FLAs. Fish organs were suspended in physiologic saline pelleted and cultured in non-nutrient agar (NNA) lawned with *Escherichia coli* and were incubated in 33 °C for 14 days. Eighteen (18) fish gills and nineteen (19) fish intestine samples presented with positive growth. Trophozoites and cystic stages of FLAs were subcultured until homogenous growth was achieved. Cells were harvested from cultured plates and DNA was extracted using Chelex resin. DNA was subjected to polymerase chain reaction using universal forward primer EukA and reverse primer EukB targeting the 18s RNA. Of the 37 plates that presented with positive amoebic growth, 9 samples showed the presence of DNAs and were sent for further purification and sequencing. Basic Local Alignment Search Tool (BLAST) results showed that protists isolated from fish organs in Lake Taal include: *Eocercomonas* (HM536152), *Colpoda steinii* (KJ607915) and *Vermamoeba vermiformis* (KC161965). The results showed that fresh-water fishes can harbour FLAs in the gut. It is proposed that freshwater reservoirs utilized for aquaculture be monitored for the presence of FLAs and

* Corresponding author.
E-mail address: gmilanez@feu.edu.ph (G.D. Milanez).

http://dx.doi.org/10.1016/j.exppara.2017.09.009

extensive study be conducted on the pathogenicity of bacterial endosymbionts and infecting viruses to its mammalian and non-mammalian host.

1. Introduction

Free living amoebae (FLAs) are considered widespread in nature and therefore called ubiquitous. Habitation of FLAs extends from aquatic reservoir to terrestrial places (Scheikl et al., 2014). According to the World Health Organization (WHO), members of the genera *Acanthamoeba*, *Naegleria* and *Balamuthia* are known to infect humans, and are considered medically important due to the fatal disease they cause to their hosts. These organisms are free living in nature but are considered parasitic once they enter the hosts.

Among the water dwelling FLAs, *Acanthamoeba and Vermamoeba* are considered to be the most represented genera in the group (Fouque et al., 2015). Unlike *Naegleria* sp. that causes primary amoebic meningoencephalitis (PAM) and *Acanthamoeba* that causes granulomatous amoebic meningoencephalitis (GAE) which are both considered fatal to the host are both isolated in freshwater, *Vermamoeba* sp. isolation was successful in soil sources in El Hierro Island, Canary Islands (Reyes-Batlle et al., 2016) where a high percentage of isolates were recorded. *Vermamoeba* sp. has also been reported to survive even in extreme temperatures and was isolated from snow samples collected in Mount Teide, Tenerife, Canary Islands, Spain (Reyes-Batlle et al., 2015). This FLA was as well reported to thrive in hospital water, which increases the possibility of contaminating and infecting humans, especially those who are immunocompromised and may lead to a more severe or even fatal outcome. The ability of this FLA to thrive in different types of environment makes it a potential public health threat.

Reports in an Iran study on contact lenses and paraphernalia for the same have shown that *Vermamoeba* sp. could be a causative agent of keratitis (Hajaialilo et al., 2015), and a study conducted on the same year demonstrated the isolation of this protozoan in dust and biofilm samples collected from different areas of an ophthalmology ward (Lasjerdi et al., 2015). *Vermamoeba* sp. has been reported to harbour important bacterial pathogens such as *Legionella* (Park, 2016) which causes legionnaires disease, the multi-drug resistant opportunistic pathogen *Stenotrophomonas maltophilia* (Pagnier et al., 2015). Other bacterial endosymbionts have also been identified such as *Mycobacterium chelonae* from *Vermamoeba* isolates from the nasal swab of an HIV/AIDS patient in Peru (Cabello-Vilchez et al., 2014). Also, *Vermamoeba* has been demonstrated to harbour giant viruses like Faustovirus (Bou Khalil et al., 2016), and Faustovirus-like Asfarvirus (Temmam et al., 2015). These literature clearly demonstrate the public health risk that *Vermamoeba vermiformis* pose not only in terms of protozoan infection but also the possible complications of which its bacterial endosymbionts and viruses it carries may confer to the host which may be potentially fatal particularly if an individual is immunocompromised. Interesting, studies have shown that *Vermamoeba* sp. has the potential to resist disinfectants, a simulated study on dental unit waterlines have demonstrated its resistance to varying concentrations of calbenium, oxygenal 6 and sterispray (Costa et al., 2016).

Vermamoeba sp. has found its way into being implicated in food contamination as it has been isolated in the gastrointestinal tract and feces of pigs which plays a role of being a source for zoonotic food-borne contamination (Chavette et al., 2016). Furthermore, *Vermamoeba* sp. has also found its way to our kitchen as it has been isolated from dishcloths along with other protozoans with *Vermamoeba* sp. being the most common isolate (Chavette et al., 2014). These literature are important sources of how *Vermamoeba* sp. can affect the gut microbiome not only in terms again of protozoan infection but also on bacterial colonization which its bacterial endosymbionts can confer not to mention the cascade of immune response the endogenous antigens from its viral residents may produce upon replication in a host cell.

The Philippines is geographically surrounded by large bodies of water and these include rivers, bays, gulfs, waterfalls, swamps and lakes. Majority of its lakes are a considerably important aquatic resource that provides food and livelihood to the localities. Lake Taal, which is located in Batangas, is one of the largest lakes after Manila de Bay and Lake Lanao. It has several fish cage farms that cultures and supplies the country with freshwater fishes such as *Sardinella tawilis* (tawilis), *Chanos chanos* (milkfish), and *Oreochromis niloticus* (tilapia). *Oreochromis niloticus* (tilapia) is the third most common freshwater fish produced in the Philippines. It is one of the highly preferred cultured fish amongst Filipinos of all social classes as it serves as a cheaper substitute for beef, pork, chicken and other animal protein. In 2014, the fish cages of region IV-A supplied 64,537 *Oreochromis niloticus* as reported by the Bureau of Fisheries and Aquatic Resources (BFAR, 2008). However, in spite of increasing demand of fishes, productivity in fish farming is not constant due to some factors such as human exploitation, environmental changes and parasitic infections. Fishes are prone to diseases like parasitism. They can also be good hosts for parasite multiplication that can be unsuitable for human consumption. Parasitism effects may vary depending upon the intensity of worm burden and pathogenicity that would result to fish kills (Salcedo et al., 2009). To date, there are no current reports and study conducted in the country whether fishes poses a potential reservoir for FLAs and whether they can be a possible cause for transmission for FLAs to humans.

2. Materials and methods

2.1. Sample collection and processing

A total of 75 *Oreochromis niloticus* were collected from three fish cages in selected municipalities surrounding Lake Taal, Batangas namely: Agoncillo, Laurel and Talisay as represented in Fig. 1. The fishes were placed in sterile plastic bags filled with lake water and with oxygen during its transport to the laboratory at the Department of Medical Technology, Far Eastern University, Manila, Philippines for further processing. Fishes were aseptically dissected upon arrival in the laboratory and fish organs (gills and intestines) were placed in sterile 3.0 ml test tubes with physiologic saline and were vortexed for 10 min. Gills and intestines were removed and remaining fluid was centrifuged at 2000 rpm for 15 min (Dykova et al., 1999). Resulting pellets were placed in previously prepared non-nutrient agar lawned with heat killed *Escherichia coli* and were placed in an incubator set at 33 °C.

2.2. Microscopic analysis

Plates were observed daily (for 14 days) using a regular

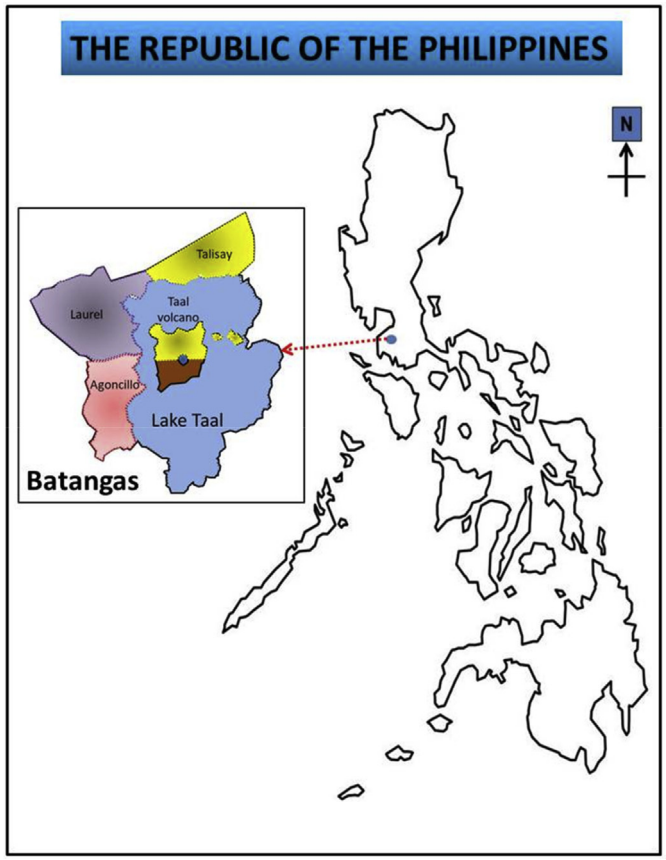


Fig. 1. Site Map of the Municipalities of Agoncillo, Laurel and Talisay around Taal Lake in the province of Batangas, Philippines.

compound microscope (Nikon Eclipse E100). The presence of FLAs was seen as clear tracks on the heat killed *E. coli* lawn produced by the feeding activity of the trophozoites or by the evidence of motile trophozoites and cystic forms. Plates that did not show any growths were kept incubated and checked for growth until fourteen days after which it was declared negative for growth (Init et al., 2010). The positive plates were left at room temperature for 1 h or overnight to allow the transformation of cyst to trophozoites prior to subculture and incubation. The surface of the agar was observed using regular compound microscope (Nikon Eclipse E100) to identify the best spot of FLA growth, marked, and the surface of the agar is cut approximately 1 × 1 cm using a sterile scalpel blade. The spot agar was placed upside down onto a new NNA- *Escherichia coli* plate and incubated at 33 °C. The step was repeated until a homogenous culture was obtained.

2.3. DNA extraction and molecular analysis

The trophozoites and cysts of each isolate of FLAs were harvested by adding approximately 5.0 ml of phosphate buffered saline (PBS) solution onto the agar surface followed by gentle scraping of the agar surface to detach the cells. The method of DNA extraction was patterned according to a previously report (Lovieno et al., 2011) using Chelex resin. Briefly, 200.0 μL of Chelex solution in 0.1% Triton X-100 and 10.0 mM Tris buffer (pH 8.0) was added to cell suspension and the mixture was vortexed for 10 s, then was centrifuged at 10,000×g for 10 s (Beckman Coulter Microfuge 16), heated to 95 °C for 20 min (AccuBlock™ Digital Dry Bath, Labnet International, NJ, USA) then finally centrifuged at 10,000×g for 20 s. The resulting supernatant was used as substrate for PCR (Lovieno et al., 2011). Substrate were further subjected to polymerase chain reaction using 18s universal primers: EukA forward primer, 5′-AACCTGGTTGATCCTGCCAGT-3 and EukB reverse primer, 5′-TGATCCTTCTGCAGGTTCACCTAC-3 as previously described (Diez et al., 2001). 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 (Medlin et al., 1988).

2.4. DNA sequencing and phylogenetic analysis

The amplicons were visualised on a 2% agarose gel stained with ethidium bromide and sent to commercial sequencing company (Macrogen, Seoul, South Korea). The presence of band at the 1100 to 1600 bp region will indicate a positive presence of free-living amoeba from the isolates (Schroeder et al., 2001). The DNA sequences were retrieved in the GenBank and analysed using Basic Local Alignment Search Tool (BLAST). Sequences obtained from the test were aligned using ClustalW of Bioedit with consideration of gaps and ambiguous sequences. Using *Naegleria* as out-group, the tree was constructed using Maximum Likelihood (ML) which is based on the best model. Bootstrap resampling were carried out with 100 replicates for ML.

3. Results

3.1. Microscopic result

A total of 18 out of 75 (24%) gill samples and 19 out of 75 (25%) intestines samples were positive for the presence of *Vermamoeba* trophozoites. Fig. 1 represents the site map where the fish samples were harvested. The percentages of FLA positivity for fish organs per sampling area (n = 25) are as follows: Agoncillo [Gills: 7 (28%); Intestine: 6 (24%)], Laurel [Gills: 5 (20%); Intestine 8 (32%)] and Talisay [Gills: 6 (24%); Intestine 5 (20%)]. Total sample positivity (n = 75) is 18 (24%) and 19 (25%) for gills and intestine respectively.

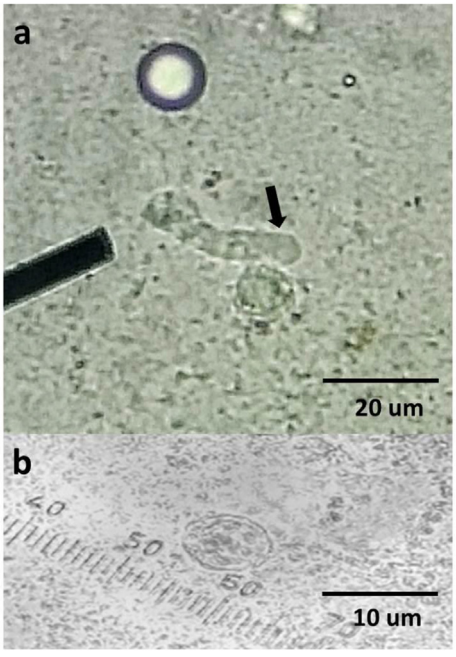


Fig. 2. Micrograph of isolated *Vermamoeba vermiformis* (MF716853). (a) Trophozoite with extension of cytoplasmic pseudopodia (black arrow) and (b) cystic stage.

Fig. 2 shows the micrograph of the isolated *Vermamoeba* sp. from the fish samples in this study. It can be observed that this *Vermamoeba vermiformis* (MF716853) isolate, labelled isolate T21-I, in its trophozoite form has an elongated shape with a single pseudopodia that extends from the posterior end of the organism. Isolate T21-I measures approximately 10–16 μm in its trophozoites form and 9–10 μm in its cystic stage with a spherical smooth walled or irregularly outlined cystic wall. Trophozoites appearance, size, movement and cystic appearance are consistent with *Vermamoeba* sp. isolates from Park (2016). Movement of trophozoites were characterized as unidirectional progressive with single pseudopodia that protrudes in the anterior part of the cell. Only a single nucleus was observed with the presences of contractile vacuoles at the posterior part of the cell. Since the organism was isolated in the intestine of the freshwater fish, it may suggest that fishes may become a potential reservoir of FLAs. Trophozoites are observed when the temperature was adjusted to 33 °C while cystic forms are seen when temperature from the incubator was changed to 30 °C.

3.2. Molecular results

The presence of eukaryotic DNA was made evident through polymerase chain reaction that targeted the 18s region using universal primers. A total of 9 samples showed the presence of eukaryotic DNA. Further DNA sequencing of PCR products revealed that three organisms present from the fish samples collected from Lake Taal namely: *Colpoda steinii* (KJ607915), *Eocercomonas* (FJ90716) and *Vermamoeba vermiformis* (MF716853). *Colpoda steinii* (KJ607915) were isolated from samples A2-I, A16-I, L12-I, T7-I, A14-G, L16-G and T7-G; *Eocercomonas* (FJ90716) were isolated from sample L16-I; and *Vermamoeba vermiformis* (MF716853) was isolated from sample T21-I. Fig. 3 shows the agarose electrophoresis result of PCR product that show bands at approximately 1600 bp suggestive of the presence of free living amoebae while Fig. 4 shows the results of the phylogenetic analysis of the isolates rooted with *Naegleria fowleri* using the MEGA 7 application.

4. Discussion

The isolation of FLA trophozoites in fish organs in this study supports the results of various papers that also outline the isolation of FLA in fish gills (Dykova et al., 1999; Salcedo et al., 2009). As *Vanella* sp., *Naegleria* sp., *Procanthamoeba* sp., *Acanthamoeba* and *Hartmanella* sp. has been observed in gills of rainbow trout *Oncorhynchus mykiss* (Dykova et al., 2010), systemic infection of the gills, kidney, intestine, pancreas and spleen with amoeba-like organisms have also been described in cultured warm water marine fishes specifically, *Trachinotus falcatus* L. from Singapore (Athanassopoulou et al., 2002). The results of this study demonstrated *Colpoda steinii* (KJ607915), *Eocercomonas* (FJ90716), and *Vermamoeba vermiformis* (MF716853) from *Oreochromis niloticus* tilapia. Furthermore, the highlight organism of this study, *V. vermiformis* which was first described by Smirnov et al. (2011), has been demonstrated to be isolated from fish organs and is able to produce tissue lesions in experimentally infected fishes (Dykova et al., 2005). Considering the studies mentioned, it would be interesting to explore the relationship of fish kill incidents commonly brought about by hypoxic events due to algal blooms, increased water temperatures, dry spells and environmental pollution and runoffs and isolation rates of FLAs particularly *V. vermiformis.*

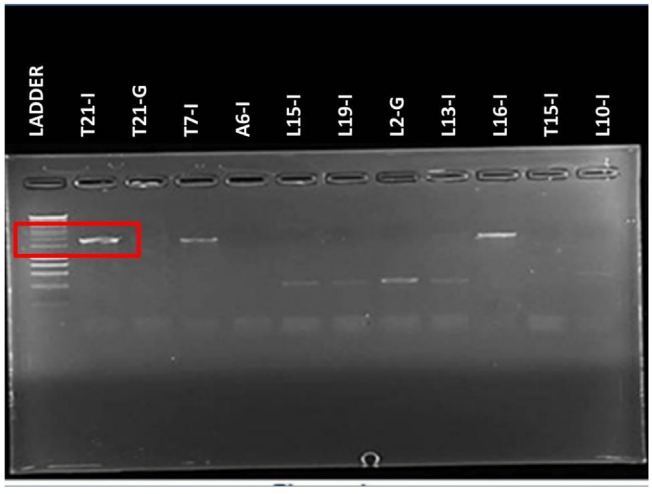


Fig. 3. Agarose Gel Electrophoresis shows a band formation at 1100–1600 bp region (red box) suggesting the presence of eukaryotic DNA from the isolates. Universal 18s RNA primers were used to detect target gene. Isolate T21-I represents *Vermamoeba* sp. (MF716853) isolate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Phylogenetic analysis of T21-I isolate of this study revealed its close relation to reference strains of *Vermamoeba* sp. from different studies that are known to harbour opportunistic facultative intracellular microorganisms. The microscopic examination of the morphology of *V. vermiformis* is an essential tool to complement its molecular analysis so as to increase the rigor of its identification (De Jonckheere et al., 2012). In addition, According to The Ohio State University *Acanthamoeba* and free-living amoeba studies website (https://u.osu.edu/acanthamoeba/studies-of-uncultured-eukaryotes-identified-as-vermamoeba/), the use of 18s RNA PCR primers is more effective in identifying *Vermamoeba* than it is in identifying *Acanthamoeba* because of the hypervariable segments present in the latter which is lacking in the former.

Several studies have demonstrated the presence of *V. vermiformis* in different environmental niches, from natural bodies of water in Poland (Adamska et al., 2014), water sources like ground water, well waters as well as in ornamental fountains in Italy (Montalbano Di Filippo et al., 2015), recreational water environments in Tehran, Iran (Nazar et al., 2012), flood water in flood affected areas during the 2011 Chiang Mai flood (Wannasan et al., 2013), in water treatment plants (Corsaro et al., 2010; Garcia et al., 2011 and Garcia et al., 2013) and in hospital water networks (Thomas et al., 2006). As far as Philippine aquaculture is concerned; this study is the first report of *V. vermiformis* found in volcanic water and in *Oreochromis niloticus* tilapia fish.

It is interesting to note the ability of *V. vermiformis* to harbour a cocktail of microorganisms such as *Legionella*, *Mycobacteria*, and viruses within its endoplasm may pose a threat to human health especially to immunocompromised individuals (Cabello-Vilchez et al., 2014). Several studies have presented a wide spectrum of other endosymbionts carried by *V. vermiformis* like *Chlamydiae* (Corsaro et al., 2010), *Pseudomonas* (Garcia et al., 2013), *Bradyrhizobium* (Thomas et al., 2006) and a tentatively named bacteria *Candidatus Nucleicultrix amoebiphila* (Schulz et al., 2014). In addition, *V. vermiformis* has been able to demonstrate resistance to biocides (Costa et al., 2016; Dillon et al., 2014) which further complicates the concern of this potentially pathogenic FLA as a carrier and melting pot for drug-resistant organisms (Pagnier et al., 2015) as well as giant viruses (Bou Khalil et al., 2016; Temmam et al., 2015) thriving and replicating in a host. It will be of great interest to be able to model the spectrum of pathogenicity these cocktail of microorganisms can confer to a suitable host both immunocompromised and immunocompetent alike and the virulence that these endosymbionts possess whilst inside its FLA host.

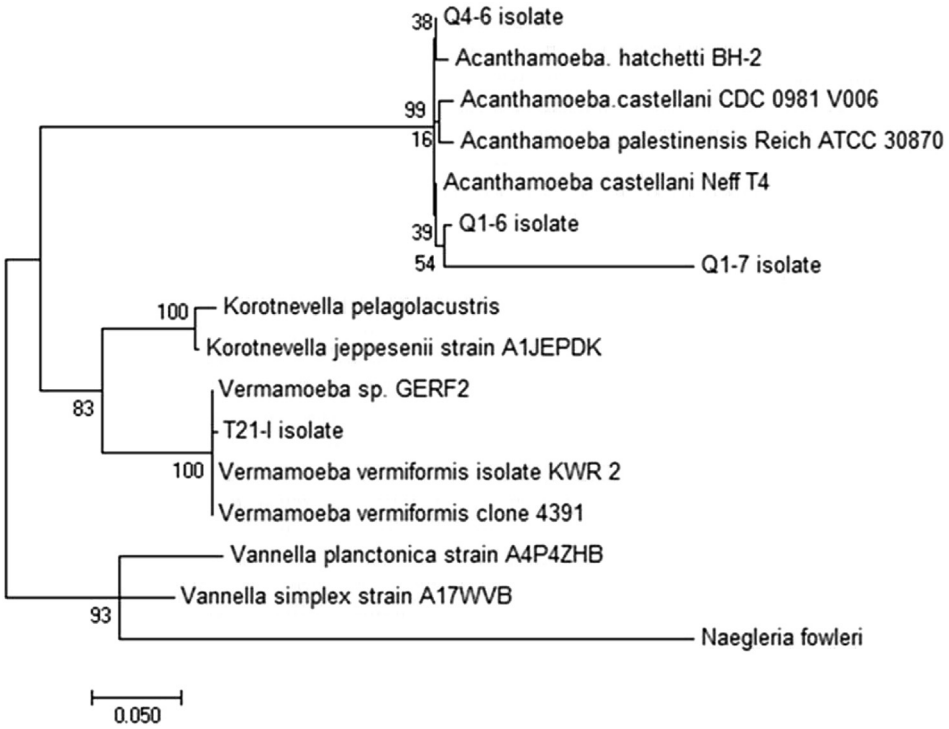


Fig. 4. Maximum likelihood tree of Isolate T21-I. Tree shows possible relationships with reference sequences of *Vermamoeba* sp. Q4-6 isolate, Q1-6 isolate and Q1-7 isolate are water sample isolates of *Acanthamoeba* sp. from Lake Taal on a separate sampling. Bootstrap showing 1000 replicates. Tree was rooted using *Naegleria fowleri* reference strain.

The presence of FLAs in aquacultures in particular fresh water fishes may pose a public health threat once consumed raw or insufficiently cooked. FLAs have been isolated from the gills of freshwater fishes, however, the isolation of FLAs from tissue lesions (Dykova et al., 2005) and in case of this study, the fish gut may suggest that fishes can act as reservoir of FLAs and that *V. vermiformis* along with other FLAs do have the ability to thrive inside the fish host and resist its immune system. Although the role and ability of *V. vermiformis* and its endosymbionts have to be able to affect the gut microbiome of humans is still unclear at this point, it can be hypothesized that it may contribute to a wide variety of localized and systemic pathogenic effects (Hajaialilo et al., 2015; Cabello-Vilchez et al., 2014; Chavette et al., 2016) and the way it will trigger the immune response of immunocompetent and immunocompromised individuals can only be predicted as possibly manageable for the former and fatal to the latter.

One thing is clear, the presence of FLA in fish organs namely the gills and intestine suggests the potential for human transmission particularly to those who work in fish farms and consumers of these produce, even those who utilize water sources for recreational purposes are at risk (Nazar et al., 2012). In addition, mammals that may frequently have contact with these infected bodies of water are at risk in contracting these FLAs and may be reservoirs that will help spread these microorganisms in otherwise FLA-free environmental systems. Consumption of poorly cooked and sometimes raw fish can place the consumers at risk. Public health education of the potential dangers of these eating habits should be disseminated to the general public, more importantly to those engaged in aquaculture. The presence of FLAs in fish gut samples strongly suggests that the same FLAs and possibly more are present in the waters of Lake Taal as well. Lake Taal is a tourist destination and is frequented by visitors during holidays. Thus, possible direct contraction of this organism is also possible by swimming in the waters of Lake Taal which has been traditionally long believed to be medicinal due to its high sulphur content. It is therefore suggested to further explore freshwater reservoirs, its resident organisms and those in close contact with it for the possible presence of FLAs particularly *V. vermiformis* and simulate models to demonstrate possible cytopathic effects and cascades of immune response in different non-mammalian and mammalian hosts.

5. Conclusions

Based on the results obtained, this study produces the first report of a potential pathogenic FLA, *Vermamoeba vermiformis* (MF716853) in fish gut in the Philippines. Results show that habitation of FLAs is not limited to water, soil, and other environmental niches but may extend to biological vectors which in turn may act as agents that can possibly transport FLAs to humans and potentially cause a wide range of immune response from cytopathic effects, spread of bacterial endosymbionts and production of endogenous antigens through viral replication. Further studies are recommended to test and observe the possible spectrums of pathogenicity in different mammalian and non-mammalian hosts.

Declaration of interest

Conflicts of interest

None.

Acknowledgements

The authors would like to thank the Department of Medical Technology and Biology Department of Far Eastern University Manila for the Technical support, Dr. Arnel Concepcion and Ms. Dulce Nisperos for the technical assistance and Dr. Windell Rivera of the Natural Science Research Institute.

References

Adamska, M., Leonska-Duniec, A., Lanocha, N., Skotarczak, B., 2014. Thermophilic potentially pathogenic amoebae isolated from natural water bodies in Poland and their molecular characterization. Acta Parasitol. 59, 433–441.

Athanassopoulou, F., Cawthorn, R., Lytra, K., 2002. Amoeba-like infections in cultured marine fishes: systemic infection in pompano *Trachinotus falcatus* L. from Singapore and gill disease associated with *Paramoeba* sp. in sea bream *Sparus aurata* L. from Greece. J. Veterinary Med. B Infect. Dis. Veterinary Public Health 49 (8), 411–412.

Bou Khalil, J.Y., Andreani, J., Raoult, D., La Scola, B., 2016. A rapid strategy for the isolation of new faustoviruses from environmental samples using Vermamoeba vermiformis. J. Vis. Exp. 4 (112) http://dx.doi.org/10.3791/54104.

Bureau of Fisheries and Acquatic Resources, 2008. Managing Aquaculture and its Impacts: a Guidebook for Local Governments. Philippines, Quezon City.

Cabello-Vilchez, A.M., Mena, R., Zuniga, J., Cermeno, P., Martin-Navarro, C.M., Gonzalez, A.C., Lopez-Arencibia, A., Reyes Batile, M., Pinero, J.E., Valladares, B., Lorenzo-Morales, J., 2014. Endosymbiotic *Mycobacteriu chelonae* in a *Vermamoeba vermiformis* strain isolated from the nasal mucosa of an HIV patient in Lima, Peru. Exp. Parasitol. 145 (Suppl. l), S127–S130. http://dx.doi.org/10.1016/j.exppara.2014.02.014. Epub 2014 Mar 1.

Chavette, N., Bare, J., Lambrecht, E., Van Damme, I., Vaerewijck, M., Sabbe, K., Houf, K., 2014. Co-occurrence of free-living protozoa and foodborne pathogens n dishcloths: implications for food safety. Int. J. Food Microbiol. 17 (191), 89–96.

Chavette, N., Lambrecht, E., Van Damme, I., Sabbe, K., Houf, K., 2016. Free-living protozoa in the gastrointestinal tract and feces of pigs: exploration of an unknown world and towards a Protocol for the recovery of free-living protozoa. Veterinary Parasitol. 225, 91–98. http://dx.doi.org/10.1016/j.vetpar.2016.06.002. Epub 2016 jun 3.

Corsaro, D., Pages, G.S., Catalan, V., Loret, J.F., Greub, G., 2010. Biodiversity of amoebae and amoeba-associated bacteria in water treatment plants. Int. J. Hyg. Environ. Health 213 (3), 158–166. http://dx.doi.org/10.1016/j.ijheh.2010.03.002. Epub 2010 Apr 18.

Costa, D., Girardot, M., Bertaux, J., Verdon, J., Imbert, C., 2016. Efficacy of dental unit waterlines disinfectants on a polymicrobial Biofilm. Water Res. 15 (91), 38–44. http://dx.doi.org/10.1016/j.watres.2015.12.053. Epub 2016 Jan 4.

De Jonckheere, J.F., Gryseels, S., Eddyani, M., 2012. Knowledge of morphology is still required when identifying new amoeba isolates by molecular techniques. Eur. J. Protistology 48 (3), 178–184.

Diez, B., Pedros-Alio, C., Marsh, T.L., Massana, R., 2001. Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic and comparison of DGGE with other molecular techniques. Appl. Environ. Microbiol. 67, 2942–2951.

Dillon, A., Achilles-Day, U.E.H., Singhrao, S.K., Pearce, M., Morton, L.H.G., Crean, S., 2014. Biocide sensitivity of *Vermamoeba vermiformis* isolated from dental-unit-waterline systems. Int. J. Biodeterior. Biodegrad. 88, 97–105.

Dykova, I., Lom, J., Schroeder-Diedrich, J., Booton, G., Byers, T., 1999. *Acanthamoeba* strains isolated from organs of freshwater fishes. J. Parasitol. 85 (6), 1106–1113.

Dykova, I., Kostka, M., Wortberg, F., Peckova, H., 2010. New data on aetiology of nodular gill disease in rainbow trout *Oncorhynchus mykiss*. Folia Parasitol. 57 (3), 157–163. http://dx.doi.org/10.14411/fp.2010.021.

Dykova, I., Pindova, Z., Fiala, I., Dvorakova, H., Machackova, B., 2005. Fish-isolated strains of *Hartmannella vermiformis* Page, 1967: morphology, phylogeny and molecular diagnosis of the species in tissue lesions. Folia Parasitol. 52 (4), 295–303.

Fouque, E., Yefimova, M., Trouilhe, M.C., Quellard, N., Fernandez, B., Rodier, M.-H., et al., 2015. Morphological study of the encystment and excystment of *Vermamoeba vermiformis* revealed original traits. J. Eukaryot. Microbiol. 62 (3), 327–337.

Garcia, A., Goni, P., Clavel, A., Lobez, S., Fernandez, M.T., Ormad, M.P., 2011. Potentially pathogenic free-living amoebae (FLA) isolated in Spanish wastewater treatment plants. Environ. Microbiol. Rep. 3 (5), 622–626. http://dx.doi.org/10.1021/es400160k. Epub 2013 Mar 13.

Garcia, A., Goni, P., Cieloszyk, J., Fernandez, M.T., Calvo-Begueria, L., Rubio, E., Fillat, M.F., Peleato, M.L., Clavel, A., 2013. Identification of free-living amoebae and amoeba-associated bacteria from reservoirs and water treatment plants by molecular techniques. Environ. Sci. Technol. 47 (7), 3132–3140. http://dx.doi.org/10.1021/es400160k. Epub 2013 Mar 13.

Hajaialilo, E., Niyyati, M., Solaymani, M., Rezaejan, M., 2015. Pathogenic free-living amoeba isolated from contact lenses of keratitis patients. Iran J. Parasitol. 10 (4), 541–546 retrieved: July 18, 2017. https://u.osu.edu/Acanthamoeba/studies-of-uncultured-eukaryotes-identified-as-vermamoeba/.

Init, I., YL, L., Arin Fadzlun, A., Foead Al, N.R., Nissapatorn, V., 2010. Detection of free living amoebae, *Acanthamoeba* and *Naegleria*, in swimming pools, Malaysia. Trop. Biomed. 27 (3), 566–577.

Lasjerdi, Z., Niyyati, M., Lorenzo-Morales, J., Haghighi, A., Taghipour, N., 2015. Ophthalmology hospital wards contamination to pathogenic free-living amoeba in Iran. Acta Parasitol. 60 (3), 417–422. http://dx.doi.org/10.1515/ap-2015-0057.

Lovieno, A., Miller, D., Lonnen, J., Kilvington, S., Alfonso, E., 2011. Extraction of *Acanthamoeba* DNA by use of Chelex resin. J. Clin. Microbiol. 49 (1), 476–477. http://dx.doi.org/10.1128/JCM.01795-10.

Medlin, L., Elwood, J.H., Stickel, S., Sogin, M.L., 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 71, 491–499.

Montalbano Di Filippo, M., Santoro, M., Lovreglio, P., Monno, R., Capolongo, C., Calia, C., Fumarola, L., D'Alfonso, R., Berrilli, F., Di Cave, D., 2015. Isolation and molecular characterization of free-living amoebae from different water sources in Italy. Int. J. Environ. Res. Public Health 12 (4), 3417–3427. http://dx.doi.org/10.3390/ijerph120403417.

Nazar, M., Haghighi, A., Taghipour, N., Ortega-Rivas, A., Tahvildar-Biderouni, F., Nazemalhosseini, M.E., Eftekhar, M., 2012. Molecular identification of *Hartmannella vermiformis* and *Vannella persistens* from man-made recreational water environments, Tehran, Iran. Parasitol. Res. 111 (2), 835–839. http://dx.doi.org/10.1007/s00436-012-2906-x. Epub 2012 Apr 4.

Pagnier, I., Valles, C., Raoult, D., La Scola, B., 2015. Isolation of *Vermamoeba vermiformis* and associated bacteria in hospital water. Microb. Pathog. 80, 14–20. http://dx.doi.org/10.1016/j.micpath.2015.02.006. Epub 2015 Feb 16.

Park, J.S., 2016. First record of potentially pathogenic amoeba *Vermamoeba vermiformis* isolated from a freshwater of Dokdo island in the East sea, Korea. Animal Syst. Evol. Divers. 32 (1), 1–8. http://dx.doi.org/10.5635/ASED.2016.32.1.001.

Reyes-Batlle, M., Niyyati, M., Martin-Navarro, C.M., Lopez- Arencibia, A., Valladares, B., Martinez-Carretero, E., Lorenzo-Morales, J., 2015. Unusual *Vermamoeba vermiformis* strain isolated from snow in Mount Teide, Tenerife, Canary Islands, Spain. Nov. Biomed. 3 (4), 189–192. http://dx.doi.org/10.22037/nbm.v3i4.1.

Reyes-Batlle, M., Wagner, C., Zamora-Herrera, J., Vargas-Mesa, A., Sifaoui, I., Gonzalez, C., Lopez-Arencibia, A., Valladares, B., Martinez-Carretero, E., Pinero, J., Lorenzo-Morales, J., 2016. Isolation and molecular identification of Vermamoeba vermiformis strains from soil sources in El Hierro island, Canary island, Spain. Curr. Microbiol. 73 (1), 104–107.

Salcedo, N., Gonzaga, E., Garuque, R., Jimenes, V., Panes, T., 2009. Detection of common parasites in freshwater fish sold at the Public Market, Kabacan, Cotabato, Philippines. USMRD J. Read. Tools 17, 147–149.

Scheikl, Ute, Sommer, Regina, Kirschner, Alexander, Remader, Alexandra, Schrammel, Barbara, Zweimuller, Irene, Wesner, Wolfgang, Hinker, Manfred, Walochnick, Julia, 2014. Free living amoebae co occurring with *Legionella* in Industrial Waters. Eur. J. Protistology 50 (4), 422–429.

Schroeder, J.M., Booton, G.C., Hay, J., Niszl, A., Seal, D.V., Markus, M.B., Fuerst, P.A., Byers, T.J., 2001. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba* from humans with keratitis and from sewage sludge. J. Clin. Microbiol. 39, 1903–1911.

Schulz, F., Lagkouvardos, I., Wascher, F., Aistleitner, K., Kostanjsek, R., Horn, M., 2014. Life in an unusual intracellular niche: a bacterial symbiont infecting the nucleus of amoebae. ISME J. 8 (8), 1634–1644. http://dx.doi.org/10.1038/ismej.2014.5. Epub 2014 Feb 6.

Smirnov, A., Chao, E., Nassonova, E., Cavalier-Smith, T., 2011. A revised classification of naked lobose amoebae (Amoebozoa: Lobosa). Protist 162, 545–570.

Temmam, S., Monteil-Bouchard, S., Sambou, M., Aubadie-Ladrix, M., Azza, S., Decloquement, P., Khalil, J.Y., Baudoin, J.P., Jardot, P., Robert, C., La Scola, B., Meddiannikov, O.Y., Raoult, D., Desnues, C., 2015. Faustovirus-like Asfarvirus in hematophagous biting midges and their vertebrate hosts. Front. Microbiol. 6, 1406. http://dx.doi.org/10.3389/fmicb.2015.01406 ecollection 2015.

Thomas, V., Herrera-Rimann, K., Blanc, D.S., Greub, G., 2006. Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network. Appl. Environ. Microbiol. 72 (4), 2428–2438.

Wannasan, A., Uparanukraw, P., Songsangchun, A., Morakote, N., 2013. Potentially pathogenic free-living amoebae in some flood-affected areas during 2011 Chiang Mai flood. Rev. do Inst. Med. Trop. São Paulo 55 (6), 411–416. http://dx.doi.org/10.1590/S0036-46652013000600007.

World Health Organization, 2003. Guidelines for Safe Recreational Water Environments. Free living Microorganisms, pp. 106–109.