


Current report on the prevalence of free-living amoebae (FLA) in natural hot springs: a systematic review

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ABSTRACT

The occurrence of potentially pathogenic free-living amoebae (FLA) in natural hot springs is considered a public health concern. FLAs are known to cause serious health outcomes to a wide spectrum of mammalian hosts. The present study aimed to provide the distribution of isolated cases of FLAs in hot springs through a systematic review process of available published articles online. Relevant studies are published between January 2010 and January 2020 involving the isolation of *Naegleria* spp., *Acanthamoeba* spp., *Balamuthia* spp., *Sappinia* spp., and *Vermamoeba* spp. in natural hot springs in the United States, South America, North America, Europe, Asia, and Africa. Articles were identified through a search of PubMed and Google Scholar databases. Out of 94 articles screened, a total of 20 articles are included in the study with consideration of established inclusion and exclusion criteria. The most common FLAs isolated in hot springs are *Acanthamoeba* spp. (134; 48.5%) and *Naegleria* spp. (127; 46.0%). Other FLAs isolated in hot springs include *Balamuthia* spp. (2; 0.7%) and *Vermamoeba* spp. (13; 4.7%). FLA in hot springs used for recreational and medical purposes is a potential source of infection. It is recommended that strict surveillance and maintenance of hot springs be implemented to prevent potential future infection.

Key words: *Acanthamoeba*, *Balamuthia*, hot springs, *Naegleria*, *Vermamoeba*

HIGHLIGHTS

- First review on the distribution of potentially pathogenic FLA in hot springs.
- Potential risk factors on FLA occurrence in hot springs.
- Occurrence of *Balamuthia mandrillaris* in hot springs.
- Occurrence of *Vermamoeba vermiformis* in hot springs.

INTRODUCTION

Hot springs are considered the main attractions to both local and foreign tourists in a geographical setting. The origins of hot springs may be categorized into different types depending on how they are formed. Some hot springs emanate from volcanic parts from the bottom of the ocean floor (McCall 2013). To add, surface waters of hot springs may be classified into three: they are considered coming from geysers; some originated from fumaroles, while others are created from cold gas seeps (Simmons 2021). Regardless of how hot springs are formed and how they are classified in terms of origin, they offer both medical and biological significance. Medically, hot springs are known to relieve musculoskeletal problems (Vaidya & Nakarmi 2020). Although there is no direct evidence on the medical effectiveness of hot springs, documentation showed a great number of microbial enzymes isolated from these water sources that may explain the partial, although still inconclusive, medicinal effects to the body (Akanbi *et al.* 2019). In a biological aspect, hot springs are home to several thermophilic organisms such as prokaryotes of different types (Castenholz 2009; Dodds & Whiles 2010), viruses (Wirth *et al.* 2021), nematodes (Poinar 2015), and a group of protozoans known as free-living amoebae (FLA) (Marciano-Cabral & Cabral 2007; Visvesvara 2010).

FLAs are considered thermophilic organisms that are normally found in freshwater sources (Izumiyama *et al.* 2005; Milanez *et al.* 2020a). This group of protozoans can survive even in high-temperature environments (Rohr *et al.* 1998). FLAs are

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known to cause severe and fatal health outcomes to humans in consequence of infection (Król-Turmińska & Olender 2017). The accidental inhalation of contaminated waters suggests the primary route of infections. The World Health Organization has named four genera considered medically important to humans: *Naegleria* spp., *Acanthamoeba* spp., *Balamuthia* spp., and *Sappinia* spp. (WHO 2003). Of the four genera, *Naegleria* and *Acanthamoeba* are known to be isolated from thermal water sources such as hot springs, to which infection can cause fatal brain conditions termed as primary amoebic meningoencephalitis (PAM) and granulomatous amoebic encephalitis (GAE), respectively (CDC 2017). To add, *Acanthamoeba* spp. can cause a debilitating eye infection known as *Acanthamoeba* keratitis (AK) once the cornea is exposed to contaminated water sources (CDC 2017). In the past, isolation of FLAs in hot springs has been documented in several studies (Sheehan *et al.* 2003; Lekkla *et al.* 2005).

The presence of these organisms in recreational waters such as hot springs, along with the fatal outcomes that result from the subsequent infection, has made the isolation of FLAs in hot springs public health importance. To date, isolation studies of FLAs in hot springs are fragmented, which warrants further investigation. This study aimed to provide the distribution of case isolation of possible FLAs that thrive in hot springs through a systematic review process of available published articles online.

METHODS

Literature search strategy

Studies involving the isolation of *Naegleria* spp., *Acanthamoeba* spp., and *Vermamoeba* spp., in natural hot springs in the United States, South America, North America, Europe, Asia, and Africa were searched systematically in PubMed and Google Scholar databases between 2010 and 2020 to provide an updated prevalence on the occurrence of FLAs in natural hot springs. The search term used to obtain the relevant studies were 'hot springs', '*Acanthamoeba*', '*Naegleria*', and '*Vermamoeba*'. To maximize the number of included studies and to prevent any missing studies during the searches of the main database, the reference list of accepted articles was searched for any additional articles to be included.

Eligibility criteria

The inclusion criterion for this study was the isolation of any FLA in natural hot springs regardless of the genus. The exclusion criteria, however, were: (1) reviews and mini-reviews, (2) studies of isolation of FLA in humans and animals, (3) case reports, and (4) articles not written in English. Screening of articles was done in two rounds, the first round of screening was done by six authors (M.R.L.F., X.R.S.D., J.A.O., K.S.V., J.-A.S.M.G., and M.S.R.), while the final screening of accepted articles was performed by one author (G.D.J.M.).

Data extraction

Data such as country location, year of study, isolated organism, type of sample used, percent of isolated organisms, methods used for identification, title, and authors were extracted from the included articles for the purpose of standardization. Data extraction was done by six authors (M.R.L.F., X.R.S.D., J.A.O., K.S.V., J.-A.S.M.G., and M.S.R.) and was checked by one author for accuracy (G.D.J.M.).

RESULTS

Search results

A total of 94 articles are screened using the key terms in this study (Figure 1). Nine articles initially removed as duplicate titles, and a further 65 articles were excluded from the list due to the following reason: 12 articles are review articles on FLAs, 3 are classified as case reports, 2 articles focused on genotyping of FLAs from other environmental sources, 14 articles isolated FLAs from other environmental sources, 3 articles declared no organism identified, 23 articles are studies that did not include hot springs as sample sources, 7 articles that have ambiguous data in abstract further do not have full text available, and 1 article written in a foreign language other than English. There are four articles, however, that are retrieved from the references of the screened articles and were added to the main number of screened articles.

Characteristics of included articles

The articles included in this study represented three continental territories namely: Asia, the Americas, and Europe. Nine of the articles from Iran, five articles are from Taiwan, two articles from Malaysia, while Brazil, Italy, Mexico, and Switzerland have all one article (Table 1). Among the reporting countries in this study, Iran has the most reported isolated FLAs with 86

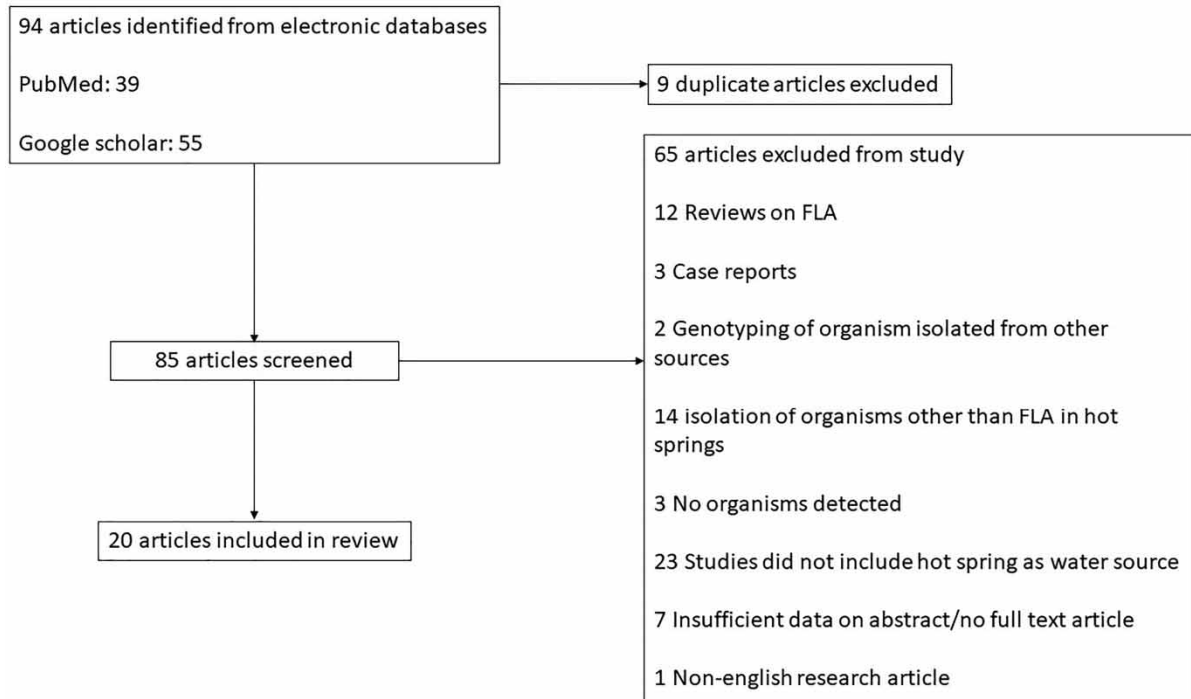


Figure 1 | Flow diagram of study selection process for including articles in the study.

Table 1 | List of accepted articles involving the isolation of FLAs in hot springs and their count per country

Country	No. of articles accepted	References
Brazil	1	Fabres <i>et al.</i> (2016)
Iran	9	Feiz Haddad <i>et al.</i> (2019) Solgi <i>et al.</i> (2012a) Latifi <i>et al.</i> (2020) Latifi <i>et al.</i> (2017) Badirzadeh <i>et al.</i> (2011) Solgi <i>et al.</i> (2012b) Dodangeh <i>et al.</i> (2018) Javanmard <i>et al.</i> (2017) Niyiyati <i>et al.</i> (2015)
Italy	1	Di Filippo <i>et al.</i> (2017)
Malaysia	2	Mohd Hussain <i>et al.</i> (2019) Latiff <i>et al.</i> (2018)
Mexico	1	Lares-Jiménez <i>et al.</i> (2018)
Switzerland	1	Gianinazzi <i>et al.</i> (2010)
Taiwan	5	Tung <i>et al.</i> (2013) Kao <i>et al.</i> (2012a) Ji <i>et al.</i> (2014) Kao <i>et al.</i> (2012b) Huang & Hsu (2010)

isolated FLAs in hot springs, Taiwan ranked second with 83 isolations, followed by Malaysia with 46 isolated FLAs, Mexico with 32 isolated cases, Brazil with eight, and finally Switzerland with six isolated FLAs. The geographical distribution and number of cases per country are illustrated in [Figure 2](#). The three most common isolated FLAs in hot springs in this study

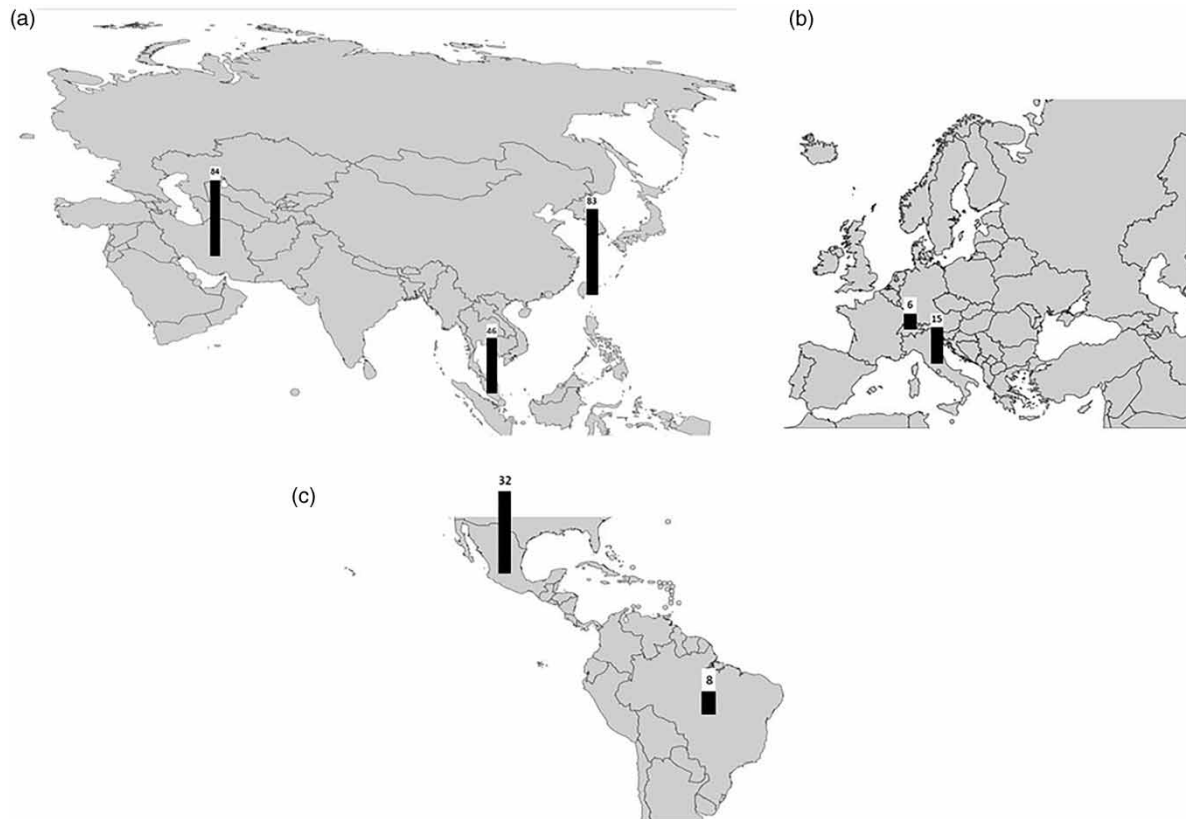


Figure 2 | Geographical distribution of included articles in the study and the number of reported cases per country (a) Asia (Iran, Malaysia, and Taiwan), (b) Europe (Italy and Switzerland), and (c) The Americas (Brazil and Mexico).

are *Acanthamoeba* genotype 4, *Naegleria australiensis*, and *Naegleria lovaniensis* (Figure 3). There are 18 articles that discuss risk factors and their potential correlation to the occurrence of FLAs in hot springs; these factors include water pH, water temperature, turbidity, salt content, and season (Table 2).

DISCUSSION

The occurrence of pathogenic FLA, especially in aquatic environments for recreational use such as hot springs, poses a major public health concern. Considering that fatal FLA infections in humans are acquired through swimming or contact with contaminated water sources as presented with recent cases (Hamaty *et al.* 2020). From another perspective, the identification of contaminated water sources (including hot springs) and patient history may serve as life-saving information in providing the proper therapeutic regimens (Vargas-Zepeda *et al.* 2005; Yadav *et al.* 2012; Chomba *et al.* 2017). In this study, it is important to note the occurrence of three established pathogenic FLAs namely: *Acanthamoeba* spp., *Naegleria* spp., and *Balamuthia* in the sampled hot springs. These FLAs are known to cause fatal health outcomes to mammalian hosts (Khan 2006; Visvesvara *et al.* 2007; Siddiqui & Khan 2012).

Occurrence of *Acanthamoeba* in hot springs

Acanthamoeba spp. is widely distributed in the environment and has been isolated in several niches (Khan 2006). Furthermore, its ability to exist as both an opportunistic and a non-opportunistic pathogen, the ability to internalize other microorganisms such as bacteria and viruses, has preceded its ill repute in a public health perspective (Schuster & Visvesvara 2004; Iovieno *et al.* 2010; Fukumoto *et al.* 2016). Several outbreaks of *Acanthamoeba* infection have been reported in the past which are all associated with the contact and use of contaminated water sources (Johnston *et al.* 2009; Verani *et al.* 2009; Yoder *et al.* 2012a, 2012b). Here, *Acanthamoeba* genotypes T4 and T15 are the most occurring with an isolation percentage of 59.7 and 22.3%, respectively, from the total of 134 *Acanthamoeba* isolated from the different hot springs. *Acanthamoeba*

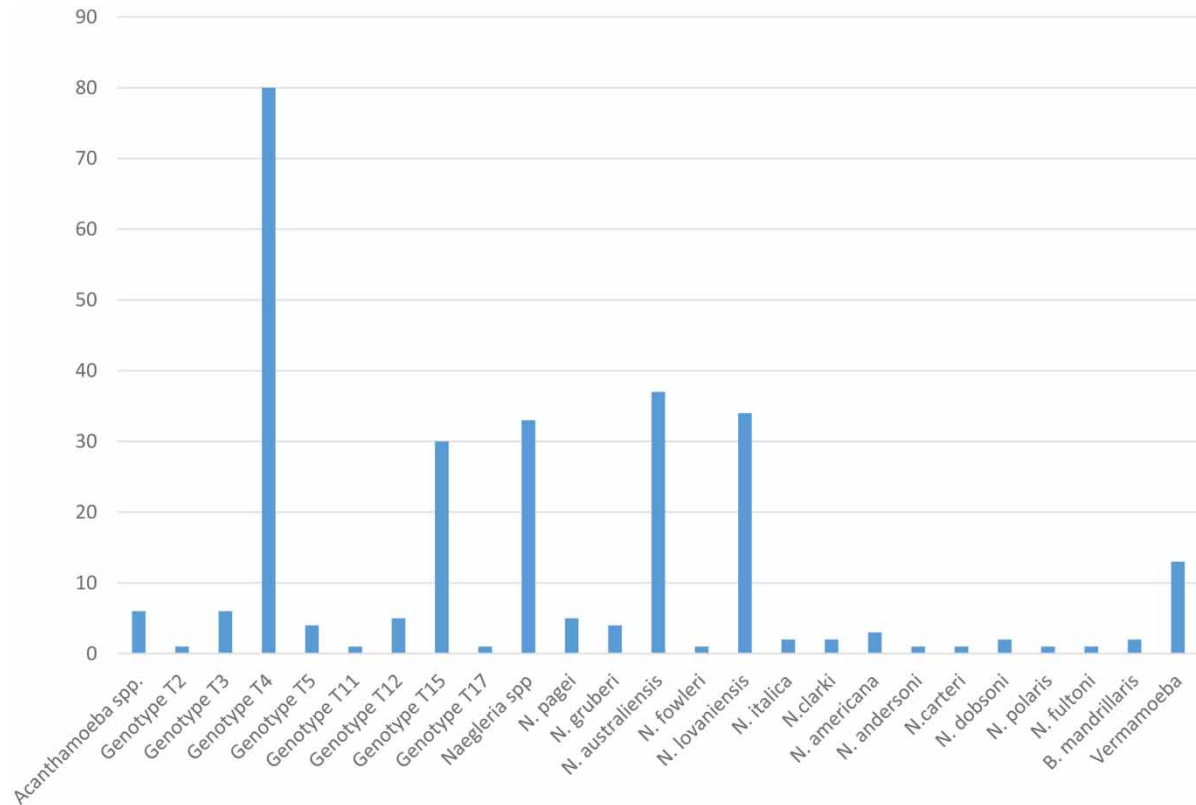


Figure 3 | Common FLA isolated in natural hot springs between 2010 and 2020 showing *Acanthamoeba* genotype T4 as the most occurring type of FLA followed by *Naegleria australiensis*.

genotype T4 is considered as one of the leading causes of *Acanthamoeba keratitis*, described as a debilitating eye infection of contact lens wearers and non-contact lens wearers (Yu *et al.* 2004; Martín-Pérez *et al.* 2017; Jercic *et al.* 2019) and GAE (Khan 2006). On the other hand, *Acanthamoeba* genotype 15 has not been an established pathogen as compared with T4 genotypes. Furthermore, this genotypic classification has been associated as an environmental isolate for some time (Booton *et al.* 2005). The recent full sequencing of *Acanthamoeba* T15 leads to its establishment in the *Acanthamoeba* phylogenetic tree classification (Corsaro *et al.* 2017) and the potential existence of pathogenic and nonpathogenic species or strains within the genus *Acanthamoeba* as suggested by some studies (Howe *et al.* 1997; Milanez *et al.* 2020a); the capacity to induce pathogenic effects of T15 and other isolated genotypes from hot springs should be taken into perspective. Although there is much debate on whether the other genotypes presented in this study are indeed pathogenic, much is needed to be done in terms of establishing their capacity to induce the disease through different *in vitro* assays and animal model testing.

***Naegleria* spp. in hot springs**

PAM is considered a fatal condition caused by organisms belonging to the genus *Naegleria* (Visvesvara *et al.* 2007). *Naegleria* spp. was first described by Fowler in 1965 in an Australian patient suffering from encephalitis (Fowler & Carter 1965). After its first description, the Centers for Disease Control and Prevention (CDC) has recorded a few cases worldwide and categorized it to be a rare human condition (CDC 2017). PAM is initiated by the organism by accessing the olfactory bulb and then crawls up to the cribriform plate which leads to the brain where the organisms cause a necrotic-like condition (Jarolim *et al.* 2000). Infection with *Naegleria* is primarily acquired through swimming in waters contaminated and eventual accidental inhalation of water (Craun *et al.* 2005). However, other routes of infection may exist as documented by some studies (Yoder *et al.* 2012a, 2012b). Hot springs included in this study revealed a high prevalence of *Naegleria* isolation, particularly 13 species which include *N. australiensis* (37 isolates), *N. lovaniensis* (34 isolates), *N. pagei* (5 isolates), *N. gruberi* (4 isolates), *N. fowleri* (1 isolate), *N. italica* (2 isolates), *N. clarki* (2 isolates), *N. americana* (3 isolates), *N. andersoni* (1 isolate), *N. carteri* (1 isolate), *N. dobsoni* (2 isolates), *N. polaris* (1 isolate), and *N. fultoni* (1 isolate). There are a total of 47 known species of *Naegleria*

Table 2 | List of potential list factors tested in included articles and relationship with isolation of FLAS

Risk factors	Observed outcomes	References
Temperature pH Season	High isolation in spring and winter; alkaline pH	Di Filippo <i>et al.</i> (2017)
Water type pH Temperature	Isolates from acidic water and temperature above 40 °C	Solgi <i>et al.</i> (2012a)
pH Temperature	Isolates observed from alkaline water	Latifi <i>et al.</i> (2020)
Temperature pH	High <i>Naegleria</i> isolation in alkaline water	Tung <i>et al.</i> (2013)
Temperature pH	High <i>Naegleria</i> isolation in alkaline water	Kao <i>et al.</i> (2012a)
Temperature pH	Significant correlation with pH and temperature	Ji <i>et al.</i> (2014)
Temperature	Water temperature was 45.5 °C	Lares-Jiménez <i>et al.</i> (2018)
Temperature	Water temperature between 43 and 46 °C	Badirzadeh <i>et al.</i> (2011)
Temperature	Water temperature significant for FLA growth	Mohd Hussain <i>et al.</i> (2019)
Temperature pH	Water pH acidic and temperature ranges from 32 to 70 °C	Solgi <i>et al.</i> (2012a)
Temperature pH	<i>Acanthamoeba</i> growth not affected by temperature and pH	Dodangeh <i>et al.</i> (2018)
Temperature pH	Alkaline water support growth of <i>Acanthamoeba</i>	Kao <i>et al.</i> (2012b)
Temperature pH	Slightly acidic to neutral pH; Temperature range of 30–34 °C; free chlorine was measured but not significant	Fabres <i>et al.</i> (2016)
pH Temperature	pH was neutral to slightly alkaline for positive samples with temperature between 38 and 40 °C	Javanmard <i>et al.</i> (2017)
Temperature	Between 34 °C and 40 °C	Gianinazzi <i>et al.</i> (2010)
Turbidity pH Environmental setting	Soil enclosure of hot spring promotes growth, high correlation with turbidity. No correlation with pH and temperature	Latiff <i>et al.</i> (2018)
pH Temperature	<i>Balamuthia mandrillaris</i> growth observed in acidic water and temperature between 32 and 42 °C	Latifi <i>et al.</i> (2016)
pH Temperature Turbidity Salt content	No significant correlation from water parameters but observed growth with sodium carbonate containing samples compared with sodium bicarbonate	Huang & Hsu (2010)

but so far, only *N. fowleri* has been considered to be pathogenic to humans (De Jonckheere 2011). Although this is the case, several studies have shown the capacity of other species belonging to this genus to induce pathogenic effects in murine models (Simeon *et al.* 1990; Schuster 2002); moreover, recent studies have been conducted to gain more insight into the molecular relationship between pathogenic and nonpathogenic *Naegleria* spp. and to establish the closeness of the organisms in a genomic level and eventually in pathogenicity factors (Liechti *et al.* 2018). With this in mind, it is important to consider the presence of both pathogenic and nonpathogenic species, especially in a common shared recreational water source such as hot springs in order to prevent potential outbreaks such as that in the Czech Republic (Karanis *et al.* 2007).

Report on the occurrence of *Balamuthia mandrillaris* and *Vermamoeba vermiformis* in hot springs

B. mandrillaris is considered a natural occurring FLA often associated with soil (Cope *et al.* 2018). This FLA has been the cause of fatal amoebic encephalitis that is known to infect a wide spectrum of mammalian hosts (Rideout *et al.* 1997; Kinde *et al.* 1998; Finnin *et al.* 2007; Hodge *et al.* 2011). Since the first report of *B. mandrillaris* caused encephalitis in the US involving primates (Visvesvara *et al.* 1990), several human case infections have been reported since then, but unlike PAM and GAE caused by *Naegleria* and *Acanthamoeba*, *B. mandrillaris* display a much longer course of clinical progression as observed from human cases (Deol *et al.* 2000; Katz *et al.* 2000; Krasaelap *et al.* 2013). The isolation of a natural soil occurring FLA in hot springs provides evidence of the capacity of *Balamuthia* to thrive even in places it does not normally inhabit. This increases the chances of fatal encounters with this FLA, especially when it thrives in water sources frequented by people. While *Balamuthia* causes a fatal infection, *V. vermiformis*' pathogenicity has yet to be established. Although a number of studies suggest that this FLA may not have a direct causality to diseases, it instead exists as a reservoir for other pathogenic microorganisms that exist as endocytobionts within its cytoplasm (Delafont *et al.* 2018; Masangkay *et al.* 2018; Scheid 2019). To add, *V. vermiformis* was recently isolated from the intestine of a freshwater fish; this further provides evidence of the capacity of *V. vermiformis* to proliferate within biological reservoirs in the environment (Milanez *et al.* 2017). The occurrence of both *B. mandrillaris* (Latifi *et al.* 2016) and *V. vermiformis* (Solgi *et al.* 2012a) in hot springs as well as evidence that these FLAs can further persist and adapt growth in the said environment can be considered as a public health concern that needs to be taken into perspective.

Potential factors affecting the growth of FLA in hot springs

The occurrence of FLAs in different aquatic and biological matrices has been an interest to several researchers in the field of FLA study. Factors such as water type, pH, temperature, season, and to some extent, the presence and absence of heavy metals are a few of the parameters that are investigated to see whether there is a direct relationship between the presence and absence of FLA in a given environment. Here, we listed in Table 2 the common parameters that were used to provide support to the occurrence of FLAs in hot springs. Five of the studies suggest that alkaline pH would promote the growth of FLAs in particular *Acanthamoeba* and *Naegleria* (Kao *et al.* 2012a, 2012b; Di Filippo *et al.* 2017; Latifi *et al.* 2020). To further argue, a current study showed that water sources considered as natural habitats for FLAs such as swimming pools and tap water have relatively neutral to alkaline pH (Kulthanan *et al.* 2013). Although this is the case, results from other studies would suggest otherwise where FLAs are isolated despite having an acidic environment (Solgi *et al.* 2012b; Latifi *et al.* 2016). Whether or not pH is a factor for FLA growth, there is evidence based on the results of the presented studies that there exists a correlation between these two variables, but until concrete statistical proof has been presented, this perspective is still a topic for debate. What is clear with the included studies is that the high water temperature in the sampling sites has clearly influenced the growth of FLAs, and this has been further given evidence by different studies (De Jonckheere *et al.* 1975; Milanez *et al.* 2019).

Isolated FLAs in hot springs and other freshwater sources

The possibility of having FLA-specific isolates in a given environmental spectrum is a question that has yet to be answered. Based on the current study, the majority of the isolated FLAs in hot springs belongs to the genus *Acanthamoeba* and *Naegleria*. Although this is the case, it is important to note that, in the case of *Acanthamoeba*, 12 genotypes (T1, T6, T7, T8, T9, T10, T13, T14, T16, T18, T19, and T20) are not reported to have been isolated in hot spring settings included in this study. Genotypes T1 and T10 are important due to their ability to cause GAE; genotypes T6 and T11 are documented genotypes that cause AK (Stothard *et al.* 1998; Walochnik *et al.* 2000; Khan *et al.* 2002). Having been said, further investigation is needed for the elucidation of what makes certain strains appear in an environmental setting or are there potential influences that affect the presence of FLAs. One possible explanation is the factor of aquaculture. Some studies have reported on the

potentiality of freshwater fishes to act as reservoirs and enable FLAs to proliferate in freshwater sources (Franke & Mackiewicz 1982; Milanez *et al.* 2017). While this is true in the case of freshwater sources such as lakes and rivers, it may not be the reason for water sources such as reservoirs and dams where the presence of aquaculture is unlikely. For this reason, pH, sufficient chlorination, and temperature would play an important part in the proliferation of certain FLA species in the said environments as presented in the case in Philippine dams (Milanez *et al.* 2020b). Whatever the reason thereof, further characterization studies are needed to explain the occurrence and nonoccurrence of certain FLA types in a given setting.

CONCLUSION

The existence of potentially pathogenic FLA in hot springs used for recreational and medical purposes is considered a public health concern. The anthropogenic activity in hot springs may be a potential source of future infection due to contact with contaminated waters. Here, we have provided the potential FLAs that may be present and were isolated from different hot springs in different geographic locations. Among the potential factors that most likely promote FLA growth in hot springs as observed from the gathered articles in this study, pH and temperature are more likely to be considered to affect the presence of FLAs in hot springs. It is the aim of this study to provide an overview of the FLA burden isolated from hot springs and to provide the possible factors that contribute to FLA proliferation in natural hot springs. It is highly recommended that strict surveillance and proper maintenance of hot springs be implemented. Lastly, it is important to consider the regular monitoring of both pH and temperature of public and private hot springs to assure the safety of those who frequent these facilities and eventually prevent future infections.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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