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Journal of Water & Health

IWA PUBLISHING

Journal of Water and Health Vol 21 No 9, 1342 doi: 10.2166/wh.2023.186

Molecular characterisation and potential pathogenicity analysis of *Acanthamoeba* isolated from recreational lakes in Peninsular Malaysia

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ABSTRACT

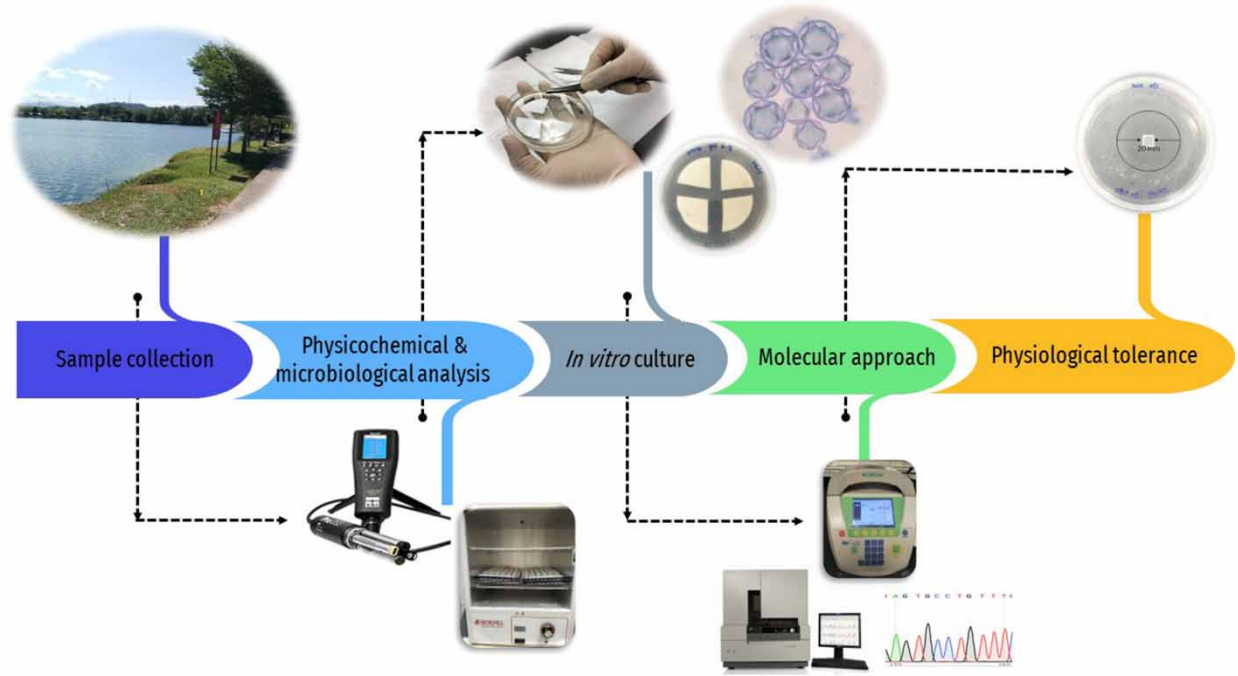
The present study aims to identify the *Acanthamoeba* genotypes and their pathogenic potential in three recreational lakes in Malaysia. Thirty water samples were collected by purposive sampling between June and July 2022. Physical parameters of water quality were measured *in situ* while chemical and microbiological analyses were performed in the laboratory. The samples were vacuum filtered through nitrate filter, cultured onto non-nutrient agar and observed microscopically for amoebic growth. DNAs from positive samples were extracted and made to react with polymerase chain reaction using specific primers. Physiological tolerance tests were performed for all *Acanthamoeba*-positive samples. The presence of *Acanthamoeba* was found in 26 of 30 water samples by PCR. The highest rate in lake waters contaminated with amoeba was in Biru Lake (100%), followed by Titiwangsa Lake (80%) and Shah Alam Lake (80%). ORP, water temperature, pH and DO were found to be significantly correlated with the presence of *Acanthamoeba*. The most common genotype was T4. Temperature- and osmo-tolerance tests showed that 8 (30.8%) of the genotypes T4, T9 and T11 were highly pathogenic. The presence of genotype T4 in habitats related to human activities supports the relevance of this amoeba as a potential public health concern.

Key words: *Acanthamoeba*, genotype, lake, Malaysia, physiological tolerance

HIGHLIGHTS

- Pathogenic *Acanthamoeba* detection in recreational lakes in Peninsular Malaysia.
- Higher *Acanthamoeba* detection rates were attributed to oxidation-reduction potential, dissolved oxygen, temperature and pH.
- First evidence of thermo- and osmo-tolerant *Acanthamoeba* T4, T9 and T11 detection in recreational lakes, Peninsular Malaysia.

GRAPHICAL ABSTRACT



1. INTRODUCTION

The opportunistic parasite *Acanthamoeba* is classified as a cosmopolitan free-living amoeba (FLA) and inhabits various environments, such as freshwater accumulations, damp and wet soil, sewage, drinking water, cooling towers and contact lens storage containers (Fanselow *et al.* 2021). *Acanthamoeba* species are distinguished at the genus level based on their varying trophozoite and cyst characteristics, particularly their double-walled cysts morphology (Sente *et al.* 2016). Initially, the morphology of *Acanthamoeba* species comprises three distinct classes (I, II and III) with over 25 nominal species (Pussard & Pons 1977). Nevertheless, the proposed morphological-based classification is imprecise in distinguishing *Acanthamoeba* species (Alves *et al.* 2000). Moreover, it is challenging to classify a cyst based on its morphological characteristics since the varying morphologies depend on the medium employed (Balczun & Scheid 2017). With advanced research, the species and genotype of *Acanthamoeba* have been widely identified using molecular approaches, which offer the most precise *Acanthamoeba* classification (Azizan & Yusof 2021).

The advanced classification of *Acanthamoeba* using the 18S ribosomal RNA gene (18S rRNA) was pioneered by Stothard *et al.* (1998). *Acanthamoeba* strains that showed differences in the 18S rRNA gene region were less than 5% and classified as a single genotype (Siddiqui & Khan 2012). This genotyping technology facilitates the documentation of strain occurrence in the environment or clinical samples and for pathogenicity purposes (Maciver *et al.* 2013). To date, the *Acanthamoeba* genus has been classified into 23 genotypes (T1–T23) (Rayes-Battle *et al.* 2022), with only a few of these genotypes pathogenic (Nagyova *et al.* 2010). The T4 isolate is the predominant genotype in environmental samples, followed by T1, T2, T3, T5, T6, T10, T12, T15 and T18 (Basher *et al.* 2018; Kalra *et al.* 2020).

Acanthamoeba cyst environmental reservoirs can rapidly spread the parasite to humans and other mammals (Lass *et al.* 2014) since they are found in seawater, thermal waters, surface waters, dam lakes and chlorinated swimming pools. The majority of genotypes known to date have been reported to infect at least one human (Azizan & Yusof 2021). Specifically, the T4, T5, T6, T11 and T15 have been identified as AK-causing genotypes that may cause health risks to humans if exposed to environmental waters contaminated with *Acanthamoeba* cysts (Lorenzo-Morales *et al.* 2015). They were also found to cause *Acanthamoeba* keratitis (AK) in non-contact lens individuals (Juárez *et al.* 2018). Therefore, a fast and effective method to identify this amoeba from environmental sources is crucial for diagnosing and treating AK.

Meanwhile, numerous scientific approaches and tools have been developed to evaluate water pollutants (Dissmeyer 2000), which determine various parameters, including pH level, turbidity, conductivity and heavy metals. According to Onichandran *et al.* (2013), these parameters are associated with the prevalence of waterborne parasites and influence their proliferation. *In situ* measurements of physical parameters, such as total dissolved solids (TDS), dissolved oxygen (DO), temperature (°C), turbidity, salinity (Sal), conductivity and pH level, are essential for indicating nutrient availability and describe the water quality relative to Malaysia's Interim National Water Quality Standard (INWQS) (DOE 2010). In addition, chemical and microbiological parameters, such as total coliform, chemical oxygen demand (COD), sulphate nitrate levels and the presence of *Escherichia coli*, provide vital information on water contamination, which may influence the occurrence of *Acanthamoeba* growth. Although *Acanthamoeba* may not directly utilise the nutrients in the water, other bacteria absorb them as their food source and lead to other health risks (Azlan *et al.* 2016).

Malaysia has diverse water sources, most of which are exploited for human activities. For instance, the majority of the population utilises recreational water resources for leisurely pursuits. As a result, they are highly exposed to the unintended splashing of *Acanthamoeba*-contaminated water in the face or bruises, allowing for rapid transmission and potentially contracting *Acanthamoeba*-causing diseases (Bunsuwansakul *et al.* 2019). In 2010, Ithoi *et al.* isolated *Acanthamoeba* from various swimming pools in Kuala Lumpur and Petaling Jaya, Selangor, Malaysia. Similarly, Onichandran *et al.* (2013) reported 100% isolation of *Acanthamoeba* from two artificial recreational lakes near Petaling Jaya. Moreover, the molecular characterisation of *Acanthamoeba* genotypes has identified for the first time the presence of pathogenic (T3, T4 and T15) and non-pathogenic (T5, T11, T17, T18 and T20) genotypes from recreational hot springs and marine waters in Peninsular Malaysia (Mohd Hussain *et al.* 2019, 2022).

Mitigating potential infections of *Acanthamoeba* is crucial, given their ability to thrive in diverse environments and the risk of spreading from anthropogenic activities. However, there is limited information regarding the occurrence of *Acanthamoeba* in recreational lakes and the existence of potentially pathogenic *Acanthamoeba* in this country. Therefore, the present study aimed to perform molecular characterisation of *Acanthamoeba* and evaluate their potential pathogenicity in three recreational lakes in Peninsular Malaysia. Briefly, physiological and microbiological water quality parameters were assessed to aid in interpreting the distribution of *Acanthamoeba* in the lake water. Positive samples were then grown and underwent advanced molecular analysis for species identification. Physiological tolerance assays were also utilised to determine the pathogenic potential of the isolates. Based on Sustainable Development Goal 6: Clean Water and Sanitation, the collected data could supplement the current baseline information on recreational lakes and implement practical policies for protecting and restoring water-related ecosystems.

2. MATERIALS AND METHODS

2.1. Sample collection and water quality assessment

A total of 30 water samples were collected from three recreational lakes in Malaysia: (a) Biru Lake, Selangor (N: 3.2475°, E: 101.5263°), (b) Titiwangsa Lake, Kuala Lumpur (N: 3.1781°, E: 101.7065°) and (c) Shah Alam Lake, Selangor (N: 3.0729°, E: 101.5138°) (Figure 1). The purposive sampling method was carried out from June through July 2022. Approximately 1 L of water samples were collected into sterile borosilicate Schott bottles at each sampling location from surface water no deeper than 10 cm at temperatures between 29 and 34 °C. They were stored at 4 °C and transferred within 24 h to the Centre for Medical Laboratory Technology Studies, Universiti Teknologi MARA, Malaysia for subsequent analysis. Overall, 10 water samples (10 L) were obtained from each recreational lake at varied sampling locations.

A portable multi-parameter (Hanna HI9828, USA) was employed to measure the physical characteristics of the water samples *in situ*, including TDS (mg/L), water temperature (°C), pH level, DO (mg/L) and oxygen reduction potential (ORP) (mV). An ITS-manufactured portable DM-TU Digimed Turbidity Meter was also applied to measure the turbidity. Meanwhile, additional water samples were collected in 500 mL sterile borosilicate Schott bottles from each sampling location and delivered to the laboratory in chilled containers within 24 h for chemical and microbiological analysis. A Hach spectrophotometer (HACH DR 2800™, USA) was then employed to determine the chemical parameters, including COD and sulphate level, based on the Hach Method (Protocol 480, 385N and 680). Furthermore, Colilert® and Colilert Quanti-Tray/2000® (IDEXX, USA) were used to determine the total coliform and *E. coli* based on the standard most probable number (MPN) technique (Painter *et al.* 2013), where the water sample was added to liquid broth media in tenfold dilutions

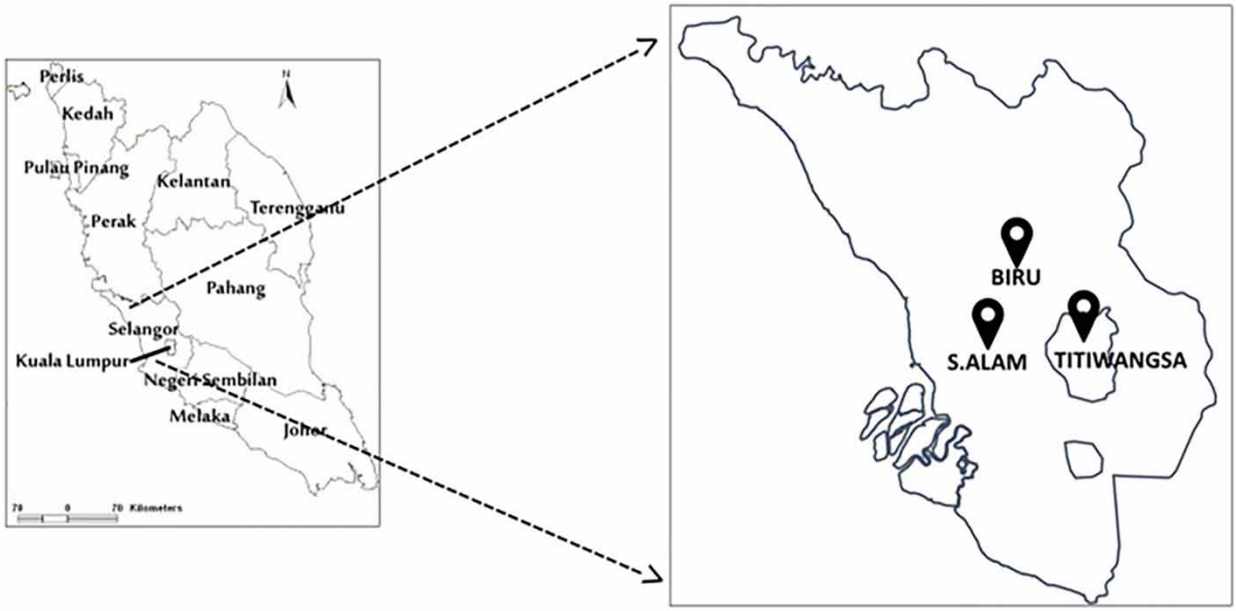


Figure 1 | A geographic map showing the location of Peninsular Malaysia and the three sampling areas involved in the present study.

and the concentration of viable microorganisms was estimated. The large and small positive wells in the Colilert Quanti-Tray/2000® were counted and the total number of positive wells was compared to the MPN table.

2.2. Isolation of *Acanthamoeba* and culture preparation

A weak manifold vacuum system equipped with a sterile bottle-top filter system and fixed with a 0.45 μm pore-size nitrate filter membrane (Gottingen, Germany) was set up to filter each 1 L of water sample at a 1.3 mL/min flow rate. After filtering, the nitrate filter membrane was flipped, cut into four equal parts and laid onto the surface of 1.5% non-nutritive agar (NNA) plates (Sigma-Aldrich A7002, USA), which is composed of Page's Amoeba Saline (PAS) solution lawned (final pH level was adjusted to 6.9) with ultraviolet (UV)-inactivated *E. coli*. The NNA plates were tightly sealed with Parafilm® before incubating for 14 days at 30 °C under 85% relative humidity (Ithoi *et al.* 2010).

The culture plates were observed daily throughout the incubation period using a bright-field microscope to detect visible morphological structures of *Acanthamoeba* cysts or trophozoites based on taxonomic criteria (Visvesvara & Schuster 2008). Subsequently, these cultures, labelled *Acanthamoeba*-positive samples, were cloned using a migration technique (Gianinazzi *et al.* 2009) and cultured at 30 °C for 3–4 days. However, cultures that did not develop morphological amoeba characteristics within 3 weeks of incubation were labelled negative. In addition, a small portion of the agar media (1 cm^2) with a minimum number of amoebae was transferred at least one to three times to fresh NNA plates lawned with UV-inactivated *E. coli* to minimise fungal growth. The transfer process was carried out based on the fungal growth rate. Note that no antibiotics were employed during the isolation process or subsequent cultivation.

2.3. Extraction of DNA, polymerase chain reaction amplification assay and sequence analysis

Prior to the DNA extraction, the grown *Acanthamoeba* cells from the cultured clones were harvested by transferring 1 mL of the PAS solution onto the surface of the agar plates. Then, a sterile L-shaped rod was used to scrape off the amoeba from each plate carefully. The amoeba-containing liquid suspension was collected in an Eppendorf tube and centrifuged in a centrifuge at 3,500 rpm for 10 min. The supernatant was collected, while the pellet was used for the DNA extraction using a QIAamp® DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Once the yield and purity of the DNA extract were measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA), the sample was stored at −20 °C until further analysis.

The polymerase chain reaction (PCR) assay was conducted to amplify the 450 bp of the 18S rRNA fragment *Acanthameoba*-specific amplimer ASA.S1 of the *Acanthamoeba* genotype. The genus-specific primers set JDP1

(5′-GGCCCAGATCGTTTACCGTGAA-3′) and JDP2 (5′-TCTCACAAGCTGCTAGGGGAGTCA-3′) were explicitly designed for *Acanthamoeba* genotyping, as previously described (Schroeder *et al.* 2001). A 50 μL PCR reaction mixture was prepared by mixing 1 μL of the DNA template (50 ng/μL) with 25 μL of TopTaq Master Mix (2X) (Qiagen, USA), 2 μL of forward and reverse genus-specific primers (each) and 20 μL of DNase-free deionised water.

The PCR protocol was set up as follows: Initial denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 1 min and extension at 72 °C for 1 min; and final extension at 72 °C for 10 min. The DNA extract sample was compared to the positive control (*Acanthamoeba castellanii* ATCC 50492) and negative control (template DNA replaced with distilled water) during each PCR run in triplicate. Subsequently, 10 μL of the PCR product was transferred in a 1.5% agarose gel (Vivantis), followed by a 100 bp DNA ladder (Biolabs, USA) as the DNA marker. Next, the gel electrophoresis was run. Once completed, the DNA fragments were identified by staining the gel with ethidium bromide (EtBr) (0.5 μg/mL) for 10 min.

The sequence analysis was conducted using a BigDye® Terminator v.3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA). Phylogenetic trees were created based on the neighbour-joining distance tree method, which generated 1,000 bootstrapped duplicates. The GenBank database was utilised to allocate the 18S rRNA gene sequences from the Blast searching and orientation using the MEGA software tool, v.11 (Mega Software, Tempe, Arizona, USA) (Tamura *et al.* 2013). Finally, the largest similarity percentage was analysed to determine the *Acanthamoeba* species.

2.4. Temperature-tolerance and osmo-tolerance tests

The temperature-tolerance and osmo-tolerance tests were performed to evaluate the pathogenic potential of the *Acanthamoeba* isolates. For the temperature-tolerance test, two sets of culture plates were prepared by soaking a small piece of NNA block containing *Acanthamoeba* cysts in the centre of each plate. Following cultivation, the initial collection of plates was incubated at 37 °C for 7 days, whereas the second collection of plates was incubated at 42 °C for 7 days. Daily inspections were performed throughout the incubation period for each plate using a bright-field microscope (400× magnification). The experiments were performed in triplicate.

For the osmo-tolerance test, small blocks of NNA containing *Acanthamoeba* cysts were cut and placed in the centre of fresh 1.5% NNA supplemented with 0.5 M or 1 M mannitol and lawned with *E. coli*. The plates were then incubated for 7 days at 30 °C to evaluate the growth. The mannitol-free NNA culture plates served as a negative control for comparison purposes. The growth performance at this stage was determined by counting the number of *Acanthamoeba* cysts or trophozoites grown approximately 20 mm from the centre of each plate and given the following score: 0 (-), 1–15 (+), 16–30 (++) and > 30 (+++) (Landell *et al.* 2013). The growth after the incubation period was evaluated similarly to the temperature-tolerance test, and the experiment was repeated three times. As a reference, *Acanthamoeba castellanii* (ATCC 50492) was grown to represent a potentially pathogenic isolate.

2.5. Statistical analysis

Statistical Package for Social Sciences (SPSS) software for Windows, version 28 (SPSS, Chicago, IL, USA) was employed to analyse the collected data. All water samples subjected to clone culture and PCR assay were evaluated descriptively to determine the prevalence rate and distribution of *Acanthamoeba* genotypes. In addition, Fisher's exact test was utilised to compare the occurrence of *Acanthamoeba* between the sampling locations. Spearman's rho correlation coefficient (r) was also applied to evaluate the correlation between the physicochemical parameters and the presence of *Acanthamoeba*. A probability (P) value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Frequency of *Acanthamoeba* occurrence in recreational lakes

The locations of the three recreational lakes (latitude and longitude) as well as the results of *Acanthamoeba* detection are presented in Table 1. Among the 30 recreational lake water samples, 26 (86.7%) samples were identified as positive using culture along with direct microscopy. The percentage of samples containing *Acanthamoeba* was highest in Biru Lake (100%), followed by Titiwangsa Lake (80%) and Shah Alam Lake (80%). The *Acanthamoeba* obtained by the culture-confirmed method were able to survive, reproduce and were usually visible within 1 week. *Acanthamoeba* revealed the presence of double-walled cysts measuring 11–25 μm (Figure 2(a)–2(c)) and trophozoites having flat shapes and spine-like

Table 1 | Detection results of *Acanthamoeba* in three recreational lakes

Sampling site	Sampling location latitude/longitude	*Acanthamoeba* percentage positivity (No. of positive/Total no.)culture-confirmed method
Biru Lake	N: 3.2475°, E: 101.5263°	100% (10/10)
Titiwangsa Lake	N: 3.1781°, E: 101.7065°	80% (8/10)
Shah Alam Lake	N: 3.0729°, E: 101.5138°	80% (8/10)
Total		86.7% (26/30)

structures (Figure 2(d)–2(e)). It is also worth mentioning that according to Fisher's exact test, there was no significant association between sampling sites and the occurrence of *Acanthamoeba* ($P = 0.315$).

3.2. Correlation between water quality parameters and the presence of *Acanthamoeba*

Correlation between the *Acanthamoeba*-positive samples based on culture-confirmed method and physicochemical parameters (DO, water temperature, pH value, TDS, ORP, turbidity, COD and sulphate) as well as microbiological parameter (*E. coli* and total coliform) are shown in Table 2. A significant positive correlation was observed between the presence of *Acanthamoeba* and oxidation-reduction potential (ORP) ($r = 0.638$; $P < 0.001$). Nevertheless, a significant negative correlation was observed between the presence of *Acanthamoeba* with water temperature ($r = -0.754$; $P < 0.001$), pH ($r = -0.575$;

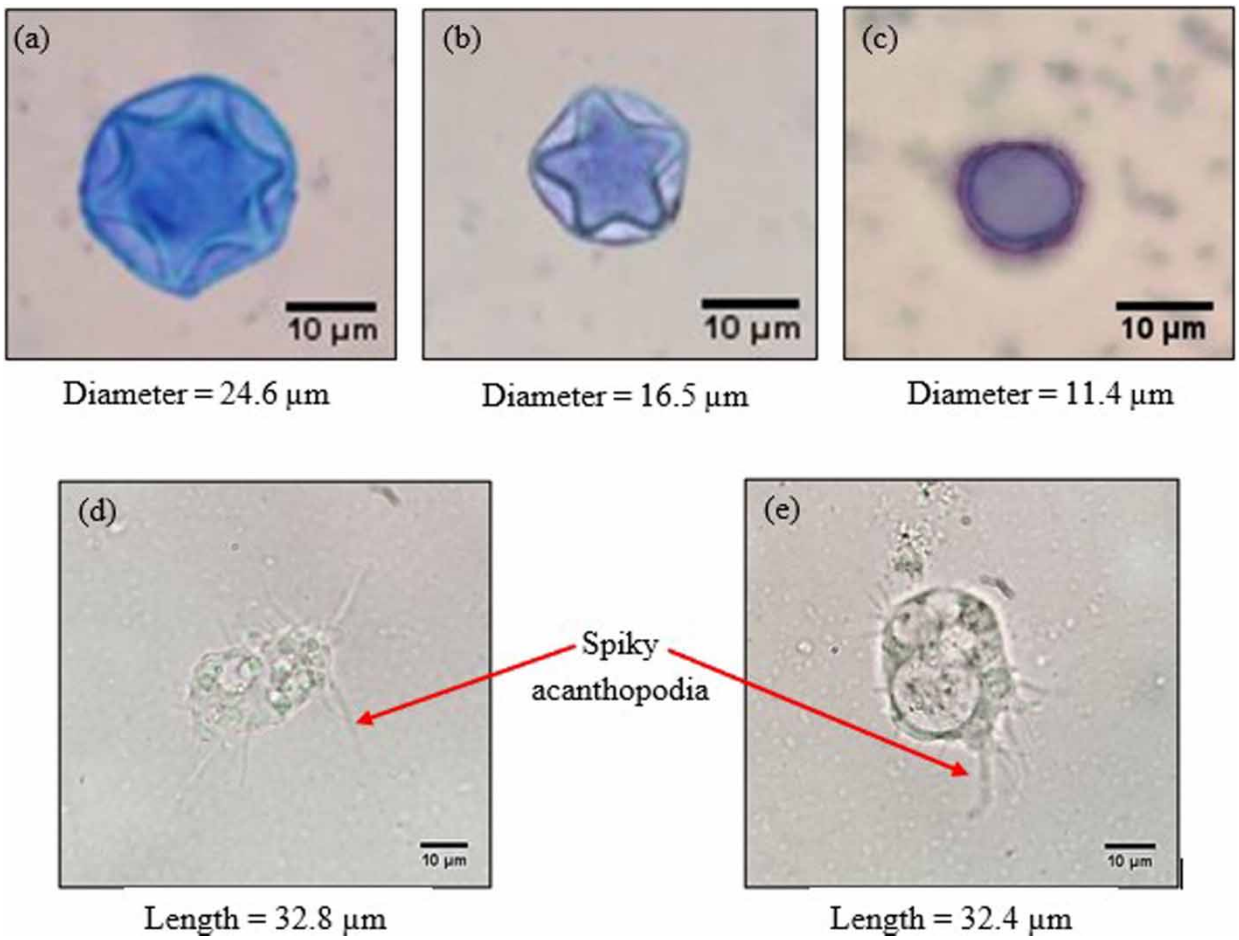


Figure 2 | Photomicrographs of representative cysts (a–c) and trophozoites (d–e) of *Acanthamoeba* isolated from recreational lake areas under 1,000× magnification.

Table 2 | Correlation coefficient (*r*) between ten water quality parameters and *Acanthamoeba* presence

Water parameter	Correlation coefficient (*r*)	Significance ($P < 0.05$)
DO (mg/mL)	−0.673	<0.001**,*
Water temperature (°C)	−0.754	<0.001**,*
pH value	−0.575	0.002**,*
Total dissolved solids (g/L)	0.259	0.201
Oxygen reduction potential (mV)	0.638	<0.001*
Turbidity (NTU)	0.279	0.379
COD (mg/L)	0.232	0.406
Sulphate (mg/L)	0.190	0.554
E. coli (MPN/100 mL)	−0.071	0.827
Total coliform (MPN/100 mL)	0.393	0.206

*Correlation is significant at the 0.05 level; **Significant at $P < 0.01$.

$P = 0.002$) and DO ($r = -0.673$; $P < 0.001$). No significant correlation was observed between the *Acanthamoeba*-positive with electrical conductivity, TDS, turbidity, COD, sulphate, *E. coli* and total coliforms.

3.3. Molecular characterisation and phylogenetic analysis of *Acanthamoeba* isolates

Acanthamoeba-positive samples detected by culture were confirmed by PCR-based detection and then sequenced to identify the species. The *Acanthamoeba* reference strains from NCBI showed a high similarity percentage (92–100%) with all 26 of the PCR products. Neighbour-joining analysis inferred the relationships between the 26 PCR products and reference strains from the NCBI GenBank, shown in Figure 3. The most frequently identified *Acanthamoeba* genotype was T4 ($n = 12$), followed by T17 ($n = 6$), T18 ($n = 4$) and then T9 ($n = 2$). Genotypes T5 and T11 were detected once, respectively (Table 3). Twelve *Acanthamoeba* isolated from Biru Lake, Titiwangsa Lake and Shah Alam Lake detected in this study were listed in genotype T4, similar to the species *A. castellanii* (MN700282) and *A. culbertsoni* (MN091854). Six *Acanthamoeba* isolated from Biru Lake and Shah Alam Lake were clustered into genotype T17, similar to the genotype *Acanthamoeba* sp. (MH791001). While four *Acanthamoeba* that were also isolated from Biru Lake and Shah Alam Lake form a cluster into genotype T18, similar to the genotype *A. byersi* (MN153028). Two *Acanthamoeba* isolated from Titiwangsa Lake were identified as genotype T9, similar to the genotype *A. astronyxis* (MN239988). Finally, two isolates T5 and T11, respectively, were isolated from Shah Alam Lake. These isolates showed similar genotypes to *A. lenticulata* (MN700283) and *A. hatchetti* (MN700300).

All of these isolates could be a possible cause of GAE and AK (Table 4). The identified genotype of *Acanthamoeba*-positive samples in the same sampling sites analysed by culture and PCR-based methods demonstrate that the *Acanthamoeba*-positive samples may include more than one *Acanthamoeba* species and genotype. The present study possibly obtained various identified *Acanthamoeba* species and genotypes through various analytical methods. The genome sequences of the isolates were submitted to GenBank under the accession numbers OQ247939–OQ247964.

3.4. Potential pathogenicity of *Acanthamoeba*

The response of the *Acanthamoeba* isolates from recreational lakes towards the temperature-tolerance and osmo-tolerance tests are shown in Table 5. Through these tolerance tests, it was found that 8 (B4, K1, K2, K8, K9, SA1, SA5 and SA10) out of the 26 isolates (30.78%) were resistant at both 37 and 42 °C temperatures including 0.5 and 1 M of mannitol. The findings also revealed that 84.62% (22/26) of the samples tested presented thermo-tolerance at 37 °C. In fact, 30.78% (8/26) of the isolates managed to overcome stressful environment at 42 °C. For the osmo-tolerance test, only one (SA4) and eight (B3, B7, K3, K5, K6, SA3, SA4 and SA6) isolates were not resistant towards 0.5 and 1.0 M of mannitol, respectively. The reference strain (*A. castellanii* ATCC 50492) used in this study also survived at 42 °C and 1 M of mannitol but with a lower number of cells than was obtained at 37 °C and 0.5 M of mannitol.

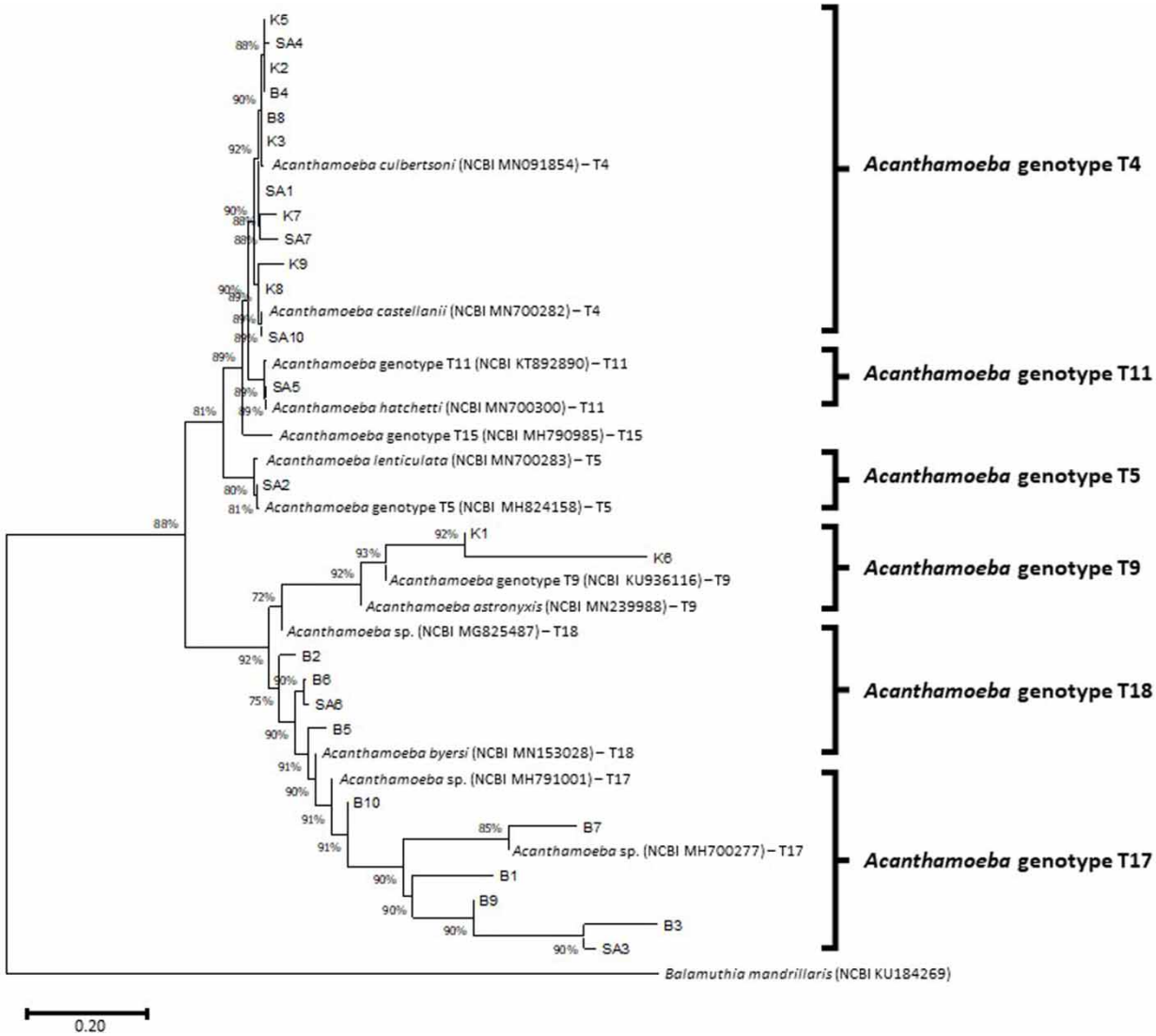


Figure 3 | Phylogenetic relationships of *Acanthamoeba* PCR products and reference strains from NCBI GenBank inferred by neighbour-joining analysis from pairwise comparisons (450 bp fragments) of the subunit 18S rRNA nucleotide sequences in three recreational lake areas. *Balamuthia mandrillaris* (NCBI KU184269) was used as an out-group. Bar index indicates the dissimilarity (0.20) among the different sequences.

Table 3 | Genotypic distribution of *Acanthamoeba* from three recreational lakes in Malaysia

	Sampling site			
Genotype	**Biru Lake**	**Titiwangsa Lake**	**Shah Alam Lake**	**Percentage (%)**
T4	2	6	4	46.2
T5	0	0	1	3.8
T9	0	2	0	7.7
T11	0	0	1	3.8
T17	5	0	1	23.1
T18	3	0	1	15.4

Table 4 | List of various *Acanthamoeba* species including *Acanthamoeba* isolated in this study and associated diseases

Genotype	Species name	Sampling site	Associated human disease
T1	*A. castellani*		Encephalitis
T2	*Acanthamoeba* sp., *A. palestinensis*, *A. pustulosa*		Keratitis and sinusitis
T3	*A. griffini*		Keratitis
T4*	*A. castellanii*, *A. culbertsoni*	Biru Lake, Shah Alam Lake and Titiwangsa Lake	Keratitis and encephalitis
T5*	*A. lenticulata*	Shah Alam Lake	Keratitis and encephalitis
T6	*A. hatchetti*, *A. palestinensis*		Keratitis
T7	*A. astronyxis*		Unknown
T8	*A. tubiashi*		Unknown
T9*	*A. astronyxis*, *A. comandoni*	Titiwangsa Lake	Keratitis
T10	*A. culbertsoni*		Keratitis and encephalitis
T11*	*A. hatchetti*	Shah Alam Lake	Keratitis and encephalitis
T12	*A. healyi*		Keratitis and encephalitis
T13	*Acanthamoeba* sp.		Unknown
T14	*Acanthamoeba* sp.		Unknown
T15	*A. jacobsi*		Keratitis
T16	*Acanthamoeba* sp.		Unknown
T17*	*Acanthamoeba* sp.	Biru Lake and Shah Alam Lake	Unknown
T18*	*Acanthamoeba byersi*	Biru Lake and Shah Alam Lake	Encephalitis
T19	*Acanthamoeba* sp.		Unknown
T20	*Acanthamoeba* sp.		Unknown
T21	*A. royreba*		Unknown
T22	*A. pyriformis*		Unknown
T23	*A. bangkokensis*		Unknown

*Isolated in the present study.

4. DISCUSSION

Acanthamoeba is one of the vastly distributed FLA in aquatic ecosystems and their potential pathogenicity in humans and animals has attracted the scientific community's interest. In response to the dramatic rise in FLA cases, numerous research has been primarily conducted on environmental samples, including lakes (Ballares *et al.* 2020; Hagosojos *et al.* 2020; Aykur & Dagci 2021). To the best of the authors' knowledge, this is the first report on the molecular characterisation and occurrence of *Acanthamoeba* in three recreational lakes in Peninsular Malaysia, routinely exploited for anthropogenic purposes. Using a combination of culture and PCR-based techniques, this study detected *Acanthamoeba* in 26 of the 30 water samples (87%). Similar research by Onichandran *et al.* (2013) and Azlan *et al.* (2016) used only morphological criteria and discovered a high prevalence of *Acanthamoeba* (100%) in designated lakes in Selangor, Malaysia. The increased contamination may be attributed to the formation of bacterial biofilm on aquatic vegetation, stones and other sediments, which stimulates a higher proliferation of *Acanthamoeba*. These protozoa and other bacteria engage in a continuous predator-prey relationship in which *Acanthamoeba* envelops and consumes innumerable bacterial colonies (Khan 2006; Sente *et al.* 2016). Conversely, Hagosojos *et al.* (2020) revealed only a 10% prevalence of *Acanthamoeba* in Lake Buhi, Philippines, which may be attributed to the use of a distinct technique in the study, where water centrifugation was applied in place of filtration prior to *Acanthamoeba* cultivation on NNA plates. The result is supported by another previous study by Gabriel *et al.* (2019), which compared various detection methods of *Acanthamoeba* in water samples. However, the study revealed that the filtration method was more effective at detecting *Acanthamoeba* than the centrifugation-based plating assay.

Table 5 | *In vitro* growth of *Acanthamoeba* isolated from recreational lakes at different temperatures and osmolarities

Sample code	Sampling site	Genotype	Temperature-tolerance assay		Osmo-tolerance assay	
			At 37 °C	At 42 °C	0.5 M mannitol	1 M mannitol
B1	Biru Lake	T17	+++	+++	+++	–
B2	Biru Lake	T18	+++	+++	+++	–
B3	Tasik Biru	T17	+++	–	–	–
B4	Biru Lake	T4	+++	+++	+++	+
B5	Biru Lake	T18	+++	++	+++	–
B6	Biru Lake	T18	+++	+++	+++	–
B7	Biru Lake	T17	+++	–	+++	–
B8	Biru Lake	T4	+++	+++	+++	–
B9	Biru Lake	T17	+++	+++	+++	–
B10	Biru Lake	T17	+++	+++	+++	–
K1	Titiwangsa Lake	T9	+++	+++	+++	++
K2	Titiwangsa Lake	T4	+++	+++	+++	++
K3	Titiwangsa Lake	T4	+++	–	+++	–
K5	Titiwangsa Lake	T4	+++	–	+++	–
K6	Titiwangsa Lake	T9	+++	–	+++	–
K7	Titiwangsa Lake	T4	+++	+++	–	–
K8	Titiwangsa Lake	T4	+++	+++	+++	++
K9	Titiwangsa Lake	T4	+++	+++	+++	++
SA1	Shah Alam Lake	T4	+++	+++	+++	++
SA2	Shah Alam Lake	T5	+++	+++	–	–
SA3	Shah Alam Lake	T17	+++	–	+++	–
SA4	Shah Alam Lake	T4	–	–	–	–
SA5	Shah Alam Lake	T11	+++	+++	+++	++
SA6	Shah Alam Lake	T18	+++	+++	+++	–
SA7	Shah Alam Lake	T4	+++	–	+++	++
SA10	Shah Alam Lake	T4	+++	+	+++	+
Reference Strain *Acanthamoeba castellanii* ATCC 50492		T4	+++	+++	+++	++

*Scores of –, +, ++ and +++ indicated for 0, 1–15, 16–30 and >30 cysts and/or trophozoites, respectively.

Meanwhile, the present study demonstrated a significant correlation between *Acanthamoeba* and ORP (mV), water temperature (°C), pH level and DO (mg/L). ORP is a measure of water's purity and ability to decompose contaminants and dead vegetation and animals. As such, a high ORP value implies a large amount of oxygen in the water. Thus, microorganisms that decompose decaying tissue and pollutants can function more efficiently in water with high ORP levels (Horne & Goldman 1994). Consequently, additional bacteria accumulate in the water leading to an increased FLA population, including *Acanthamoeba* (Sente *et al.* 2016). Despite the fact that *Acanthamoeba* is considered a thermophilic amoeba, it tends to be more prevalent in lake waters within a moderately low-temperature tolerance of 29.4–33.7 °C. The occurrence of a high trophozoite count at a low temperature in this study is consistent with previous findings, which reported an optimal growth temperature of approximately 30 °C for *Acanthamoeba* (Nielsen *et al.* 2014) as well as in other literature (Sente *et al.* 2016; Mohd Hussain *et al.* 2019). On top of that, the pH values measured in this study at 6.9–9.3 is well within the recommended national water quality standard for recreational lakes (Class IIB) (DOE 2019). This finding is similar to the most recent report by Mohd Hussain *et al.* (2022), where a significant correlation between the presence of *Acanthamoeba* and the pH level of marine water was observed. The negative correlation in the present study also indicated that lower

pH levels of lake water signify a greater likelihood of the presence of *Acanthamoeba*. Khan (2006) stated that pathogenic *Acanthamoeba* can thrive in a pH range of 4–12. A change in the DO level would be more complex regarding community interactions. Toxins cause the mortality of other organisms, which would indirectly consume oxygen and affect the survival of oxygen-producing and oxygen-consuming microorganisms. In the current study, the presence of *Acanthamoeba* increased the DO level (5.2–10.9 mg/L), which exceeds the 5–7 mg/L limit set by the Malaysian national water quality standards for recreational lakes (DOE 2019). Tsai *et al.* (2020) inferred that such a phenomenon could be related to predation activity (bacteria), indicating poor environmental and recreational lake water quality. In addition, the DO concentration may be altered due to other bacterial activities, such as heterotrophic bacterial respiration, oxygen generation by phototrophic species and other microbial metabolisms (Riedel *et al.* 2013; Sente *et al.* 2016).

Based on the molecular approach and NCBI comparison in this study, six of the partially identified *Acanthamoeba* sequences belonged to the T-genotypes, including T4, T5, T9, T11, T17 and T18, in addition to six identical previously known *Acanthamoeba* spp. (*A. castellanii*, *A. culbertsoni*, *A. lenticulata*, *A. astronyxis*, *A. hatchetti* and *A. byersi*). Only one *Acanthamoeba* species was not assigned to a specific species, possibly indicating a unique species to Malaysia. This study also revealed that the reported T4 genotype comprised two species that were 99–100% identical to *A. castellanii* and *A. culbertsoni*. This was consistent with previous local studies, which stated that the T4 genotype is frequently detected in various water sources, such as hot springs and marine waters (Mohd Hussain *et al.* 2019, 2022). The predominance of the T4 *Acanthamoeba* genotype heightens the risk of infection in humans, which aligns with the frequently reported clinical cases, especially in AK and GAE patients (Booton *et al.* 2002; Yera *et al.* 2007). According to Kao *et al.* (2014) and Ghaderifar *et al.* (2018), *A. castellanii* (T4 genotype) is responsible for over 90% of all documented AK cases. Based on this data, it is conceivable that recreational lakes in Malaysia are a significant reservoir of pathogenic *Acanthamoeba* that can transmit acanthamoebic diseases to humans.

Meanwhile, T17 (six strains) was the second most prevalent genotype among the identified *Acanthamoeba* strains. However, none of these strains matched any specific species in the NCBI database, suggesting that they may be Malaysia-specific. The presence of the T17 genotype in environmental water samples has also been reported in freshwater sources in Thailand (Nuprasert *et al.* 2010), hot springs in Malaysia (Mohd Hussain *et al.* 2019) and swimming pools in Turkey (Değerli *et al.* 2020). Genotype T17, belonging to the morphological group I, is identified as a non-pathogenic strain but rarely detected in the environment, making it a less research subject (Magliano *et al.* 2012; Diehl *et al.* 2021). T18 is another uncommon genotype discovered in Biru Lake and Shah Alam Lake samples at a prevalence rate of 15.4%. Surprisingly, this was the first detection of the T18 genotype in recreational lakes in Malaysia. So far, this genotype has been isolated from sewage and rivers (Possamai *et al.* 2018), as well as the brain and lungs (Matsui *et al.* 2018). Moreover, the isolation of the novel T18 genotype from a small number of samples suggests that this genotype may have thrived abundantly in nature. Thus, more novel *Acanthamoeba* genotypes may exist in the environment and remain to be discovered.

In contrast, T9, T5 and T11 are the genotypes with the lowest frequency in this study. Similarly, Ballares *et al.* (2020) reported the presence of *Acanthamoeba* T9 in samples collected from Seven Crater Lakes of Laguna, Philippines. The Tasik Titiwangsa-isolated genotype T9 displayed 92% homology with *A. astronyxis*. Although *A. astronyxis* was initially identified as a potential cause of a non-fatal case of human meningitis (Callicott *et al.* 1968), this is no longer believed to be the case. Nonetheless, research on *A. astronyxis* is not widely available (Magliano *et al.* 2012). Hajialilo *et al.* (2016) have linked T9 to AK infection, making it an emerging pathogen from a public health standpoint. Clusters of *A. lenticulata* (T5) and *A. hatchetti* (T11) were detected in a sample from Shah Alam Lake, but only a single isolate was identified in the current study. This confirms the presence of T5 and T11 in diverse water reservoirs, as previously reported (Todd *et al.* 2015; Dendana *et al.* 2018; Mohd Hussain *et al.* 2019; Milanez *et al.* 2020). Genotype T5 could cause keratitis (Ledee *et al.* 2009) in immunocompromised individuals and disseminated infection, as reported in a case involving a heart transplant patient (Barete *et al.* 2007). Therefore, the presence of T5 in recreational lakes may be a significant risk factor for *Acanthamoeba* infection, particularly in immunocompromised individuals. In the meantime, since the prevalence of AK is still uncommon when associated with T11, additional epidemiological research is being conducted to confirm the association between T11 and clinical cases. This genotype has thus far been isolated from clinical specimens in Iran (Niyyati *et al.* 2009).

It is difficult to determine the correlation between *in vitro* evaluation and the actual human pathogenic potential of *Acanthamoeba*, given its diverse genotypes. The profound heterogeneity has been emphasised by the response of the organism to pathogenicity experiments (Tawfeek *et al.* 2016). Notably, eight isolates (30.8%) of *Acanthamoeba* comprising the T4,

T9 and T11 genotypes were able to thrive at elevated temperatures (42 °C) and intense osmotic stress (1 M), indicating an indirect association between virulence factors with potential pathogenicity (Khan 2001). A recent study in the Philippines also reported that 47% of samples tested from two major water reservoirs (lakes and rivers) were temperature-tolerant and could flourish at 40 °C (Milanez *et al.* 2020). The ability of *Acanthamoeba* to survive under high temperatures and osmolarity makes them more potentially virulent than strains that can only exist at 30 °C (Visvesvara *et al.* 2007; Wannasan *et al.* 2009; Todd *et al.* 2015). However, the *in vitro* growth of *Acanthamoeba* isolates under elevated temperatures or intense osmotic stress is correlated to their infectiousness, as this is partially associated with their survival and adaptation ability in host tissues of mammals (Khan & Tareen 2003). It is essential to note that the T4, T9 and T11 genotypes are predominately pathogenic, which may be due to the strains being exposed to environmental stress and may be associated with other factors, such as the synthesis of heat shock proteins (HSP70) (Solgi *et al.* 2012). Contrarily, Kahraman & Polat (2022) isolated T4B and T4E genotypes from keratitis cases and found that three of the four strains exhibited zero growth at 39–41 °C and 1 M mannitol. Thus, *Acanthamoeba* tolerance to 39 °C and 1 M mannitol does not indicate its pathogenicity. It is therefore necessary to conduct a broad range of pathogenicity analyses on *Acanthamoeba* species, such as cytopathic effects to confirm the infection impact of each genotype on humans (Mohd Hussain *et al.* 2022).

5. CONCLUSION

In conclusion, this study demonstrated the presence of unique *Acanthamoeba* genotypes with varying degrees of pathogenicity in recreational lakes across Malaysia. The culture and PCR-based approaches highlighted that the T4 genotype inhabited environmental waters related to human activities and verified their potential threat to human health. Hence, precise identification of risk factors that may cause contamination should be implemented through proactive programmes designed to prevent future infections. Ultimately, substantial engagement should be exercised to raise awareness among the public regarding the hazards of potentially waterborne diseases and sufficient warning should be placed to inform the population of the significant health risk at these locations.

ACKNOWLEDGEMENTS

This study was financially supported by the Fundamental Research Grant Scheme (FRGS/1/2022/SKK0/UITM/02/13), Ministry of Higher Education, Malaysia and Geran Inisiatif Penyeliaan (600-RMC/GIP 5/3[088/2022]), Universiti Teknologi MARA, Malaysia.

DATA AVAILABILITY STATEMENT

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

CONFLICT OF INTEREST

The authors declare there is no conflict.

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First received 23 June 2023; accepted in revised form 13 August 2023. Available online 24 August 2023