ORIGINAL ARTICLE

Molecular Characterization and Physiological Tolerances of *Acanthamoeba* Isolated from Recreational River Water in Templer Forest Eco Park, Selangor: A Preliminary Study

Tengku Shahrul Anuar[1,2], Wan Naili Alia[1], Rania Farzana Azmi[1], Rosnani Hanim Mohd Hussain[1], Ratna Wahyuni[3,4]

[1] Centre for Medical Laboratory Technology Studies, Faculty of Health Sciences, Universiti Teknologi MARA, Puncak Alam Campus, Selangor, Malaysia.
[2] Microbiome Health and Environment (MiHeaRT), Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam, Selangor, Malaysia
[3] Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia
[4] Department of Health, Faculty of Vocational Studies, Universitas Airlangga, Surabaya, Indonesia

ABSTRACT

Introduction: *Acanthamoeba* species are ubiquitous free-living protozoa that can be found worldwide. *Acanthamoeba* in recreational waters is concerning because it can cause serious human health issues, particularly *Acanthamoeba* keratitis and granulomatous amoebic encephalitis. Currently, this amoeba can be classified into 23 different genotypes, labeled T1 to T23. This study targets the identification of *Acanthamoeba* genotypes and their pathogenic characteristics within Templer Forest Eco Park, Selangor. **Materials and Methods:** To conduct this research, purposive sampling was performed, resulting in the collection of ten water samples from March to April 2023. Each sample underwent filtration using a nitrocellulose membrane, followed by analysis for *Acanthamoeba* presence through cultivation and PCR utilizing specific primers. Physiological tolerance assessments were executed on all samples that tested positive for *Acanthamoeba*. **Results:** PCR testing confirmed the presence of *Acanthamoeba* in 8 out of the 10 sampled water sources. Phylogenetic analysis classified six of these samples as genotype T18, while remaining samples were identified as genotypes T4 and T17. Tolerance assessments revealed one *Acanthamoeba* strain (genotype T4) exhibited significant pathogenicity. **Conclusion:** This is the first time that *Acanthamoeba* genotypes have been reported in recreational waters in Selangor. The findings suggest that *Acanthamoeba* strains present in the river water of Selangor may pose a potential health risk to humans. Therefore, it is recommended that health authorities implement regular monitoring of river water and install warning signage to mitigate the risk of diseases linked to pathogenic *Acanthamoeba*.
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Corresponding Author:
Tengku Shahrul Anuar, PhD
Email: tengku9235@uitm.edu.my
Tel: +603-3258 4425

INTRODUCTION

Acanthamoeba has emerged as a major public health concern dueF to its abiFlity to cause devastating human infections. Although such infections are relatively rare, the consequences can be disastrous, particularly with conditions like *Acanthamoeba* keratitis (AK), which primarily affects contact lens wearers and granulomatous amoebic encephalitis (GAE), a rare but frequently life-threatening brain infection in individuals with compromised immune systems (1, 2). According to culture and polymerase chain reaction (PCR) methods, 50% of water and 100% of soil samples were contaminated with *Acanthamoeba*. Based on the sequencing data, genotypes T4 (47%), T5 (35.29%), T3 (11.76%) and T11 (5.88%) were identified in water samples (3). Certain *Acanthamoeba* trophozoites not only inflict harm but also harbor pathogenic bacteria such as *Legionella* sp., *Helicobacter pylori* and *Mycobacterium leprae*, which can resist destruction by amoebae, along with other 'amoeba-resistant microorganisms' that occur naturally in environmental water (4, 5). It has been postulated that these amoebae may act as biological incubators or reservoirs for bacteria, functioning as 'Trojan horses' that facilitate transmission to humans (6).

The identification of *Acanthamoeba* at the genus level necessitates an examination of both cysts and trophozoites, particularly the distinctive double-walled cyst morphology. Initially, the *Acanthamoeba* species were classified into three morphological categories (I, II

and III) (7), encompassing over 25 nominal species (8). Nevertheless, this classification was inconsistent due to a lack of substantial correlation between molecular typing and binomial nomenclature in various cases. A single recognized species may possess multiple genotypes, while one genotype can be associated with more than one recognized species. Moreover, the inconsistencies in strain/species clustering have been further highlighted by biochemical, growth, pathogenic traits and distribution patterns (9). Stothard et al. (10) pioneered a more advanced method for the classification of *Acanthamoeba* utilizing the 18S ribosomal RNA gene. Less than 5% of *Acanthamoeba* strains displayed variations in the 18S rRNA gene region and were classified as a single genotype (4). This genetic identification technology assists in documenting the presence of strains within environmental or clinical samples to study pathogenicity (11). To date, 23 genotypes (T1–T23) have been identified within the *Acanthamoeba* genus, with only a limited number of these genotypes recognized as pathogenic (12). The predominant genotype found in environmental samples is T4, followed by T1, T2, T3, T5, T6, T10, T12, T15 and T18 (13).

Although *Acanthamoeba* genotypes do not consistently correspond with species identified through morphological characteristics, the extensive diversity within this genus contributes to its widespread distribution and adaptability to various environmental conditions. Booton et al. (8) determined that T4 is the predominant genotype in both environmental and clinical samples during their analysis of *Acanthamoeba* genotypes. The remaining pathogenic genotypes (T2, T3, T5, T6, T10, T12, T15 and T18) were infrequently found in clinical samples (14). Nevertheless, genotyping alone does not yield sufficient insights into the pathogenicity of an isolate. Usually, a thorough description of *Acanthamoeba* is enhanced by looking at its physiological characteristics. Certain characteristics linked to its disease-causing potential exhibit resilience against protease activity, elevated temperatures above 37°C, cytopathic influences on cultured cells and high osmolarity (15). Both thermal and osmotic tolerance tests can effectively differentiate between non-pathogenic and pathogenic isolates (16). In proposing a plating assay to distinguish between pathogenic and non-pathogenic isolates, Khan et al. (17) identified that a crucial trait of pathogenic amoebae is their capacity to thrive under high osmolarity and temperature conditions. The elevated secretion of heat shock proteins in pathogenic isolates was linked to this phenomenon.

In Malaysia, the presence of *Acanthamoeba* has been extensively documented across various environmental sources, showing a substantial prevalence in water supplies, soil and indoor contexts. Studies have shown that *Acanthamoeba* contamination is alarmingly high in those lakes and rivers that attracts tourists and holidaymakers, with some locations exhibiting a 100% positivity rate (18). Artificial environments, including swimming pools and domestic tap water have shown notable contamination rates, indicating that these amoebae can endure even with routine maintenance and water treatment procedures (19). Recent research on water samples from beach revealed contamination rates ranging from 60% to 90%, with genotypes T4, T5, T11, T18 and T20 identified (20). This broad environmental distribution increases the likelihood of human exposure, particularly for contact lens users and individuals with compromised immune systems, placing Malaysia at greater risk than many other regions. However, there is still a lack of molecular data and pathogenic potential of *Acanthamoeba* isolates from Malaysia recreational river waters. Therefore, the objective of this preliminary study was to identify the *Acanthamoeba* genotypes present in a popular recreational river and assess their potential pathogenicity via physiological tolerance tests. We hypothesized that pathogenic genotype, such as T4 might be present in the recreational river water and that at least some isolates would show high thermo- and osmo-tolerance indicative of health risk.

MATERIALS AND METHODS

Sampling site and sample

Templer Forest Eco Park is situated north of Batu Cave, approximately 20 km away from downtown Kuala Lumpur, Malaysia. It is in the same rainforest area as the nearby Kanching Waterfalls and Commonwealth Forest Park. Templer Forest Eco Park is greatly beloved by enthusiasts of nature. It offers a swimming pool for visitors who prefer not to hike to reach a waterfall. They can also relax by swimming in the pool naturally filled with river water. The peaceful and calm atmosphere enables all visitors to unwind, let go of stress and refresh themselves. Ten samples of surface water were collected from ten distinct locations identified as TPMY_1, TPMY_2, TPMY_3, TPMY_4, TPMY_5, TPMY_6, TPMY_7, TPMY_8, TPMY_9 and TPMY_10. The samples were collected from March to April 2023. All sampling points were selected on the basis of accessibility and community presence, which evidenced anthroponotic activities. Water samples are often taken from the surface of a river, within 10 cm of the middle, to collect representative samples of the water's composition and characteristics. This depth allows for easy access and minimizes disturbance of the riverbed, while also capturing the flow and mixing of the water (18). One liter of water was collected in a sterile borosilicate Schott bottle at every sampling location, then kept at 4°C until being delivered within 48 hours to the Parasitology Laboratory at the Universiti Teknologi MARA, Malaysia for further examination. No permit was required to collect water from this public sampling sites. However, verbal consent was obtained from the relevant authority before the commencement of the study.

Sample filtration and cultivation of *Acanthamoeba*

The method of membrane filtration was applied to each one-liter water sample collected in a sterile bottle top filter device using a cellulose nitrate membrane filter (0.45 µm pore size) from Gottingen, Germany and a low vacuum manifold system with a flow rate of 1.3 ml/min (21). Following filtration, the membrane was reversed, partitioned into four parts and placed on 1.5% non-nutrient agar (NNA) dishes (Sigma Aldrich A7002, USA) covered with Page's amoeba saline (PAS) solution containing UV-inactivated *E. coli* K12 (ATCC 10798). The final pH of the PAS solution was then adjusted to 6.9. The dishes were carefully covered with Parafilm® and placed upside down at 30°C, with an 85% relative humidity for a maximum of 14 days (19).

Microscopic examination and culture preparation

The evaluation was carried out daily on every culture plate for a period of two weeks using a light inverted microscope (Lieca DMI3000 B, Germany) before being deemed negative following the criteria outlined by Page (22). The *Acanthamoeba* trophozoites were detected based on their irregular shape, spiny pseudopods (acanthopodia) and a single nucleus, while cysts were detected based on a double-walled structure with a wrinkled outer layer (ectocyst) and an inner layer (endocyst) that can be polygonal, spherical or star-shaped, respectively. The characteristics of the cyst and trophozoite were examined using a bright-field microscope (ZEISS Primo Star, Germany) after creating a thin smear from agar possibly containing *Acanthamoeba*. A marker pen was used to draw a circle on the plate's bottom, marking the surface of agar containing *Acanthamoeba*. Next, the plate's cover was taken off and a specific area on the agar surface was gently scraped using a coverslip. The gel was flipped over and evenly pushed onto a glass slide to be viewed under a bright field microscope at a magnification of 1,000Ч. Plates with colonies that tested positive in culture were sub-cultured ten times by moving a colony containing four to six cysts onto newly made NNA-*E. coli* plates in order to establish a homogenous cell culture. Trophozoites from individual isolates were cultured in five plates to gain 5x103 cell. Later, they were harvested to extract total DNA with QIAamp® DNA mini kit (Qiagen, Hilden, Germany).

DNA extraction, PCR amplification assay and sequence analysis

Before extracting DNA, *Acanthamoeba* cells that had been cultured were harvested by transferring 1 mL of the PAS solution onto agar plates. Next, a sterile L-shaped rod carefully scraped off the amoeba from each plate. The liquid suspension containing amoebas was placed in an Eppendorf tube and spun in a centrifuge at 3,500 rpm for 10 min. The supernatant was then removed and the pellet was utilized for DNA extraction using a QIAamp® DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instruction manual. After determining the yield and purity of the DNA extract with a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA), the sample was kept at -20 °C for future analysis.

The polymerase chain reaction (PCR) assay was conducted to amplify the 423–551 bp of the 18S rRNA fragment *Acanthamoeba*-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 (23). Next, the PCR solution was prepared with 1 µL of the DNA template (50 ng/µL), along with the PCR mixture in order to establish a total volume of 50 µL. The PCR mixture comprised 25 µL of TopTaq Master Mix (2X) (Qiagen, USA), 2 µL each of the oligonucleotide primers, along with 20 µL of DNase-free deionized water.

The PCR procedure began with an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 1 min and extension at 72°C for 1 min. Lastly, there was a final extension at 72°C for 10 min. During each PCR run in triplicate, the DNA extract sample was analyzed alongside the positive control (*Acanthamoeba castellanii* ATCC 50492) and negative control (template DNA replaced with distilled water). Following that, 10 µL of the PCR sample was transferred to a 1.5% agarose gel (Vivantis), with a 100 bp DNA ladder (Biolabs, USA) used as the DNA marker. Subsequently, the gel electrophoresis was conducted. After finishing the process, the DNA fragments were detected by staining the gel with ethidium bromide (EtBr) (0.5 µg/mL) for a duration of 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 neighbor-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) (24). Finally, the largest similarity percentage was analysed to determine the *Acanthamoeba* species.

Thermo-tolerance and osmo-tolerance tests

In this study, thermo- and osmo-tolerance physiological evaluations were performed in triplicate. The cultures cultivated on an NNA medium enriched with *E. coli* were employed in the assessment following the methodology reported previously (20). Two culture plate sets were prepared for the assays. In the thermo-tolerance evaluation, a small NNA block soaked with *Acanthamoeba* trophozoites or cysts was placed centrally on each culture plate. Subsequently, freshly prepared 1.5% NNA was overlaid with *E. coli* suspension

before incubating the cultures at 37 and 42°C. Small agar block consisting of *Acanthamoeba* cysts were sliced and positioned in the center of a fresh 1.5% NNA medium of 0.5 or 1 M of mannitol for osmo-tolerance assessment. The cultures were also overlaid with *E. coli.* The mannitol-free NNA plates were the negative control in this study.

The cultures were observed daily under a bright-field microscope at 400x magnification over a period of 5 days. The proliferation of *Acanthamoeba* was assessed by counting all cysts or trophozoites located approximately 20 mm from the center of each plate. The mean counts of each sample were recorded from triplicate measurements. Growth was quantified based on the following categories: 0 (–), 1–15 (+), 16–30 (++) and >30 (+++). At the end of the analysis, samples were classified as high (+++), low (+ to ++) or non-pathogenic (–) based on previously published criteria (25). *A. castellanii* (ATCC 50492) was used as the reference strain to confirm pathogenic characteristics.

RESULTS

Molecular characterization and phylogenetic analysis of *Acanthamoeba*

Culture-confirmed *Acanthamoeba* positive samples underwent further validation through PCR-based detection (Figure 1) and were subsequently sequenced for species identification. The *Acanthamoeba* reference strains from NCBI demonstrated a high degree of similarity (98–100%) with all eight PCR products. A neighbor-joining analysis was conducted to establish the relationship among the eight PCR products and the reference strains from NCBI GenBank, as depicted in Figure 2. The T18 genotype was the most frequently identified, occurring a total of six times. Genotypes T4 and T17 were each identified once, as indicated in Table I. Two *Acanthamoeba* isolates collected from Templer Forest Eco Park were categorized as genotype T18, which corresponds to the genotype of *A. byersi* (OQ247944 and MN153028). A single *Acanthamoeba* was classified under genotype T4, analogous to the species *A. castellanii* (MF100902). Additionally, four *Acanthamoeba* of genotype T18 and genotype T17 exhibited similarities to *Acanthamoeba* sp. (MW181633 and OQ247945). Each of these isolates holds the potential to be a source of AK and GAE (Table II). The identification of genotypes from *Acanthamoeba* positive samples across the same sampling locations examined through culture and PCR-based methods indicates that the *Acanthamoeba* positive samples may encompass multiple *Acanthamoeba* species and genotypes. This study has likely yielded various identified *Acanthamoeba* species and genotypes through diverse analytical approaches. The genomic sequences of the isolates have been archived in GenBank under accession numbers OR352420–OR352427.

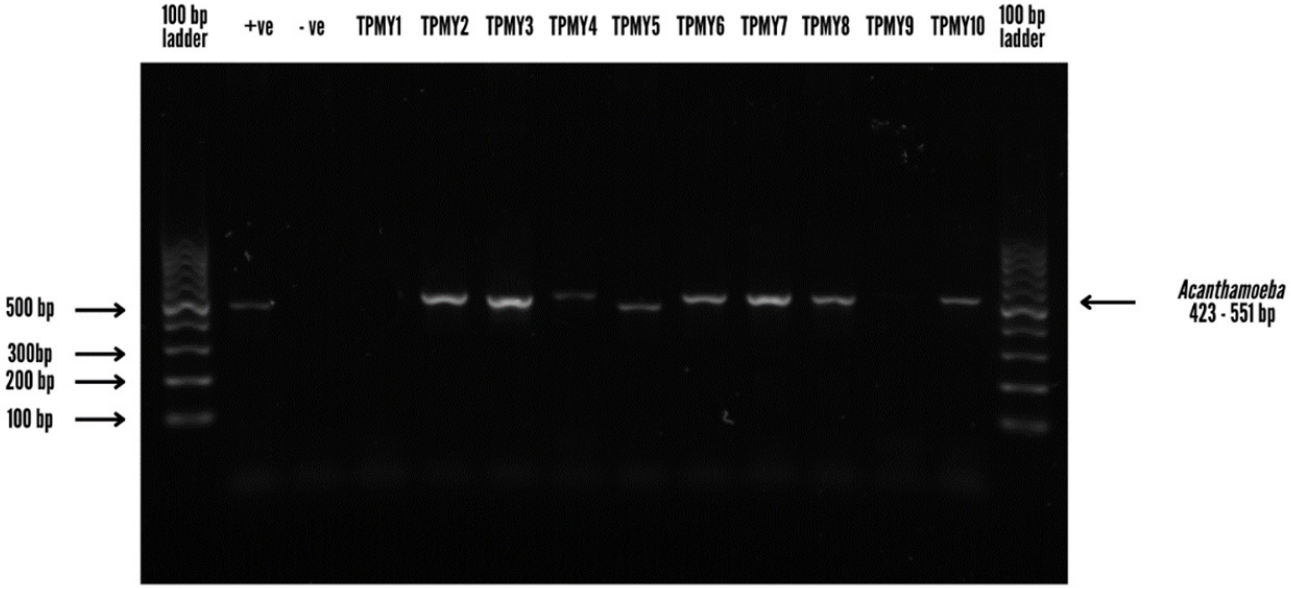

Figure 1: PCR amplification of the isolated *Acanthamoeba* strains using JDP primers. Lane 1: 100 bp ladder was used as a molecular size marker, Lane 2: positive control (*A. castellanii* ATCC 50492), Lane 3: negative control (without DNA), Lanes 5, 6, 7, 8, 9, 10, 11 and 13: positive cases with high load of *Acanthamoeba* in the samples and Lanes 4 and 12: negative samples.

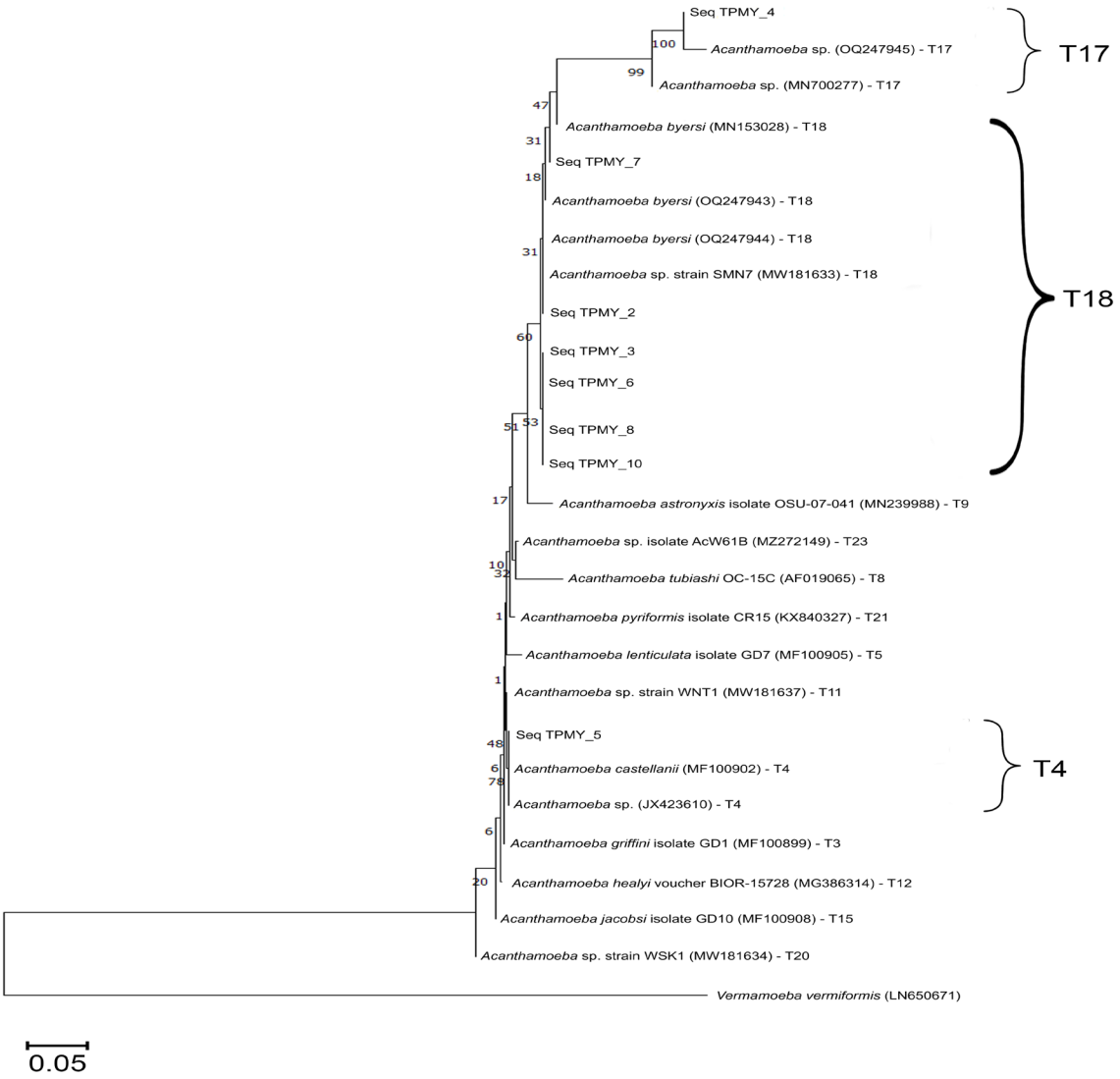

Figure 2: The phylogenetic tree of *Acanthamoeba* PCR products and reference strains from the NCBI GenBank reconstructed based on the neighbor-joining method using MEGA 11. *Vermamoeba vermiformis* (NCBI LN650671) was used as the outgroup.

Table I: List of *Acanthamoeba* isolates of the eight PCR positive samples and the reference strains with the highest homology found from the NCBI GenBank.

Isolate	Accession number	Genotype	Reference strain with highest homology	Accession number	Region of origin	Reference
TPMY_2	OR352420	T18	*A. byersi*	OQ247944	Malaysia	47
TPMY_3	OR352421	T18	*Acanthamoeba sp.*	MW181633	Thailand	40
TPMY_4	OR352422	T17	*Acanthamoeba sp.*	OQ247945	Malaysia	47
TPMY_5	OR352423	T4	*A. castellanii*	MF100902	Malaysia	47
TPMY_6	OR352424	T18	*Acanthamoeba sp.*	MW181633	Thailand	40
TPMY_7	OR352425	T18	*A. byersi*	MN153028	Austria	48
TPMY_8	OR352426	T18	*Acanthamoeba sp.*	MW181633	Thailand	40
TPMY_10	OR352427	T18	*Acanthamoeba sp.*	MW181633	Thailand	40

Table II: List of various *Acanthamoeba* genotypes and species isolated in this study and associated disease.

Genotype	Species name	Associated human disease	Reference
T4	*A. castellanii*	Keratitis and Granulomatous amoebic encephalitis	49
T17	*Acanthamoeba sp.*	Unknown	50
T18	*A. byersi* *Acanthamoeba sp.*	Granulomatous amoebic encephalitis	14

Physiological tolerances of *Acanthamoeba*

Table III presents the findings from the thermo- and osmotolerance assessments performed on *Acanthamoeba* isolates obtained from river water. The assessment revealed that one isolate (TPMY_5), representing 12.5% of the cohort, exhibited resistance to temperatures of both 37 and 42°C, as well as to osmotic challenges presented by 0.5 and 1 M mannitol. Additionally, it was found that 87.5% of the isolates demonstrated tolerance to heat at 37°C, with 50% maintaining viability under elevated temperatures at 42°C. Regarding osmotic pressure resistance, only two isolates showed susceptibility to 0.5 M mannitol. Notably, the *A. castellanii* ATCC 50492 reference strain was able to withstand conditions of 42°C and 1 M mannitol, though with a reduced cell count compared to outcomes observed at 37°C and 0.5 M mannitol.

Table III: *In vitro* growth of *Acanthamoeba* isolated from river water at different temperatures and osmolarities.

Sample Code	Genotype	Score for growth[a]			
		Thermo-tolerance		Osmo-tolerance	
		37°C	42°C	0.5 M mannitol	1 M mannitol
TPMY_2	T18	+++	–	+++	–
TPMY_3	T18	+++	+++	+++	–
TPMY_4	T17	–	–	–	–
TPMY_5	T4	+++	+	+++	+
TPMY_6	T18	+++	–	+++	–
TPMY_7	T18	+++	+++	+++	–
TPMY_8	T18	+++	+++	–	–
TPMY_10	T18	+++	–	+	–
Reference Strain *A. castellanii* (ATCC 50492)	T4	++	+	+	++

[a]Scores of –, +, ++ and +++ indicated for 0, 1–15, 16–30 and >30 cysts and/or trophozoites, respectively, were seen in five microscope fields (at 100x).

DISCUSSION

Acanthamoeba species exhibit remarkable adaptability, as evidenced by their occurrence in both natural and artificial habitats (4). Consequently, exposure to *Acanthamoeba* poses potential risks to individual health. The increasing participation in river-based recreational activities raises concerns due to the associated decline in health among residents and visitors (26). This study represents the first investigation in Selangor focusing on the molecular genotyping and pathogenic characteristics of *Acanthamoeba* at Templer Forest Eco Park. The presence of *Acanthamoeba* was confirmed in 80% of the water samples collected using culture and PCR techniques, indicating a robust presence of *Acanthamoeba* species in the river water at the studied locations, which is concerning considering the human activities at Templer Forest Eco Park throughout the year. This finding is consistent with reports by Basher et al. (18), who noted a high occurrence (100%) of *Acanthamoeba* in river water across central west Malaysia. Furthermore, *Acanthamoeba* species were identified in 66% of the 100 sites surveyed during the water sample collection from public freshwater sources in Thailand (27). A recent study conducted by Salazar-Ardiles et al. (28) revealed that the *Acanthamoeba* genus was predominantly found in water and sediment samples from the Loa and Salado rivers in northern Chile. Notably, Kang et al. (29) identified the presence of *Acanthamoeba* in the Southern Han River in Korea. This suggests that the *Acanthamoeba* genus is widely disseminated in natural environments and highlights the inevitability of its detection in soil and water samples.

During the study, samples belonging to the *Acanthamoeba* genus were identified using the Acanthamoeba-specific primers, JDP1 and JDP2. A 423–551 bp fragment was amplified in eight samples, which aligns with the observations made by Salehi et al. (30), who similarly detected *Acanthamoeba* sp. with PCR products ranging from 423 to 551 bp, identified as ASA.S1. The ASA.S1 amplimer is uniquely specific to the *Acanthamoeba* genus and is present in all recognized 18S rRNA genotypes (23). Recently, Chan et al. (31) demonstrated that the primer pair JDP1 and JDP2 selectively amplifies the ASA.S1 segment of *Acanthamoeba* 18S rRNA genes, effectively excluding other closely related amoeba genera. In another study, Fuerst and Booton (32) noted that the ASA.S1 fragment has emerged as a focal point in the examination of *Acanthamoeba* isolates. Furthermore, Mohd Hussain et al. (33) indicated that the size of the amplicon utilized in the analysis of the 18S rRNA gene is crucial for the accurate identification of *Acanthamoeba* isolates.

Recent research has identified three distinct partial *Acanthamoeba* sequences (T4, T17 and T18), along with two identical sequences (*A. castellanii* and *A. byersi*), using NCBI comparisons to known *Acanthamoeba* species sequences. Additionally, five unclassified *Acanthamoeba* species were noted as distinct within the Malaysian context. The presence of the *Acanthamoeba* strain exhibiting genotype T4 raises significant health concerns for humans. In this study, the T4 genotype demonstrated high levels of homology, showing 98% and 100% to *A. castellanii*, respectively. Similar findings were reported by Huang and Hsu (34), who also detected genotype T4 in spring resort areas across Taiwan. In contrast, Hsu et al. (35) indicated that genotype T4 was the predominant genotype (75%) found in rivers and reservoirs in Taiwan. Richard et al. (36) similarly found that genotype T4 was the most frequently isolated genotype from a water treatment facility in Sarawak, Malaysia. Despite being isolated only once in this study, genotype T4 remains a significant concern as it is the leading genotype among pathogenic *Acanthamoeba* species that pose a risk to human health. Furthermore, Booton et al. (8) established that genotype T4 is often

linked to AK infections, with two isolates in their study classified as *A. castellanii.* Additionally, a majority of GAE samples identified correlated with clinical isolates connected to the T4 genotype (13).

In river water samples, genotype T18 was the predominant isolate, representing 75% of the tested specimens. Although considered rare, the T18 genotype was recently identified in Malaysian marine waters by Mohd Hussain et al. (20). Similarly, a study in Brazil documented the rare *Acanthamoeba* genotype T18 in both river and sewage waters (37). Research by Putaporntip et al. (27) also found the *Acanthamoeba* genotype T18 in Thai freshwater sources. The presence of genotype T18 in the river water of Templer Forest Eco Park raises concerns for potential *Acanthamoeba* infections, particularly GAE, as this genotype has been linked to the illness (38). Moreover, prior studies have reported the identification of *A. byersi* genotype T18 in skin and brain tissues of GAE patients who had a history of organ transplants (14). Consequently, the presence of genotype T18 in aquatic environments may lead to *Acanthamoeba* infections among individuals engaging in recreational water activities, particularly those with compromised immune systems.

This research identified a specific strain of the T17 genotype, consistent with a prior study conducted by Tanveer et al. (39), which similarly detected the T17 genotype in canal water samples from Iran. In a related investigation, the T17 genotype was found in Malaysia by Mohd Hussain et al. (21) at a recreational hot spring in Bentong, Pahang, where *Acanthamoeba* sp. was identified. Furthermore, the T17 genotype has been documented in prior study conducted in Thailand from sources such as pond water and soil (40), indicating that the T17 genotype is commonly found in environmental samples. To date, no human illnesses have been associated with the T17 genotype (4). However, several pathogenic bacteria also known associated with *Acanthamoeba*, which may play a role as possible reservoirs (4, 5). Nevertheless, recent research indicates that various non-pathogenic *Acanthamoeba* genotypes from the T7/T8/T9/T17 group may possess the potential to cause diseases, highlighting the importance of further studies (41).

The enduring tropical climate in Malaysia provides an advantage for environmentally resilient cyst forms of free-living amoebae (FLA). Chan et al. (42) proposed that a higher proportion of *Acanthamoeba* in Malaysian environments may exhibit natural thermal resistance. This finding aligns with the identification of 7 out of 8 samples (88%) of *Acanthamoeba* isolates from river water, which have demonstrated the ability to survive elevated temperatures of 37°C, despite the optimal growth temperature being around 30°C (43). Importantly, the capability to thrive at 37°C is a significant indicator of a microorganism's potential to induce disease, as the human eye's temperature is approximately 34°C (44). Additionally, it is noteworthy that only one *Acanthamoeba* isolate (TPMY_5) of the T4 genotype was able to survive at a high temperature of 42°C. This strain's resilience to extreme environmental conditions may be attributed to several factors, including the synthesis of heat-shock proteins (HSP), particularly HSP70 (45). This hypothesis is supported by earlier research conducted by Pйrez-Serrano et al. (46), which investigated the survival and release levels of HSP60 and HSP70 in various *Acanthamoeba* species in response to abrupt temperature changes. The study indicated that elevated levels of HSPs could reflect their role in enhancing tolerance to host stressors and/or in the virulence of the species.

Osmotic tolerance assessments indicated that six out of eight isolates (75%) exhibited resistance to 0.5 M mannitol, whereas only one isolate demonstrated resistance to 1 M mannitol. The ability to endure high osmotic pressure in response to increased mannitol concentrations has been linked to the growth of amoebas, particularly during their parasitic phase in the corneal epithelium (4). The *Acanthamoeba* samples were adept at maintaining their cellular structure and functionality, employing effective mechanisms to regulate osmotic balance. Notably, only the TPMY_5 isolate (T4 genotype) was resistance to both 0.5 M and 1 M mannitol; in contrast, isolates TPMY_2, TPMY_3, TPMY_6, TPMY_7, TPMY_8 and TPMY_10 (T17 and T18 genotypes) were unable to survive in 1 M mannitol. This variation in osmoregulatory capabilities may explain their proficiency in sustaining the equilibrium of water and solutes within their cells in response to fluctuating external osmotic conditions. The disparities in osmoregulation across different *Acanthamoeba* genotypes could correlate with genetic differences and adaptations to their specific environments.

Collecting water from rivers for *Acanthamoeba* studies has limitations due to the complex and dynamic nature of river ecosystems and the potential for contamination from various sources. These limitations include (i) limited sampling sites and (ii) cost and time constraints. Rivers are large and complex, making it challenging to collect representative samples that accurately reflect the overall *Acanthamoeba* distribution. Moreover, conducting extensive sampling and analysis of river water can be costly and time-consuming especially for large-scale studies. Therefore, ten locations in the present study are all in one section of the river, which were selected on the basis of accessibility and community presence (evidenced anthroponotic activities). Despite these limitations, river water remains an important source of information for understanding the epidemiology and ecology of *Acanthamoeba*, particularly when combined with other data sources and research methods.

CONCLUSION

In summary, the present study detected multiple *Acanthamoeba* genotypes (T4, T17 and T18) in a popular recreational river, with one isolate (genotype T4) showing high thermo-tolerance indicative of the capacity to cause serious infection. This suggests that visitors to such water bodies could be at risk of *Acanthamoeba*-related illnesses (such as keratitis), albeit no cases have been reported yet.

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REFERENCES

1. Fanselow N, Sirajuddin N, Yin XT, Huang AJ, Stuart PM. *Acanthamoeba* keratitis, pathology, diagnosis and treatment. Pathogens. 2021; 10:323. doi: 10.3390//pathogens10030323.
2. Khurana S, Sharma C. *Acanthamoeba* keratitis: different hues of the parasite. Indian Journal of Medical Microbiology. 2022; 40:150–151. doi: 10.1016/j.ijmmb.2021.10.010.
3. Mahmoudi MR, Maleki M, Zebardast N, Rahmati B, Ashrafi K, Sharifdini M, Karanis P. Identification of *Acanthamoeba* spp. from water and soil of public parks in the north of Iran. Journal of Water and Health. 2022; 20:1604–1610. doi: 10.2166/wh.2022.193.
4. Siddiqui R, Khan NA. Biology and pathogenesis of *Acanthamoeba*. Parasites & Vectors. 2012; 5:6–18. doi: 10.1186/1756-3305-5-6.
5. Wahyuni R, Astari L, Iswahyudi, Paling S, Adriaty D, Kurniawati S, Aulia S, Rahmatari B, Prakoeswa CR, Agusni I, Izumi S. *Mycobacterium leprae* survival inside *Acanthamoeba* sp. isolated from water source in leprosy endemic area, Indonesia. Journal of Tropical Life Science. 2024; 14: 269-276. doi:10.11594//jtls.14.02.07
6. Janda WM. Amoeba-resistant bacteria: their role in human infections. Clinical Microbiology Newsletter. 2010; 32:177–184. doi: 10.1016/j.clinmicnews.2010.11.001.
7. Pussard M, Pons R. Morphologie de la paroi kystique et taxonomie du genre *Acanthamoeba* (Protozoa, Amoebida). Protistologica. 1977; 13:557–559.
8. Booton GC, Visvesvara GS, Byers TJ, Kelly DJ, Fuerst PA. Identification and distribution of *Acanthamoeba* species genotypes associated with nonkeratitis infections. Journal of Clinical Microbiology. 2005; 43:1689–1693. doi: 10.1128/JCM.43.4.1689-1693.2005.
9. Khan NA, Tareen NK. Genotypic, phenotypic, biochemical, physiological and pathogenicity-based categorisation of *Acanthamoeba* strains. Folia Parasitologica. 2003; 50:97–104. doi: 10.14411/fp.2003.017.
10. Stothard DR, Schroeder-Diedrich JM, Awwad MH, Gast RJ, Ledee DR, Rodriguez-Zaragoza S, Dean CL, Fuerst PA, Byers TJ. The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. Journal of Eukaryotic Microbiology. 1998; 45:45–54. doi: 10.1111/j.1550-7408.1998.tb05068.x.
11. Maciver SK, Asif M, Simmen MW, Lorenzo-Morales J. A systematic analysis of *Acanthamoeba* genotype frequency correlated with source and pathogenicity: T4 is confirmed as a pathogen-rich genotype. European Journal of Protistology. 2013; 49:217–221. doi: 10.1016/j.ejop.2012.11.004.
12. Reyes-Batlle M, Sifaoui I, Rodriguez-Exposito RL, Pinero JE, Lorenzo-Morales J. New insight in Acanthamoeba. Pathogens. 2022; 11:609. doi: 10.3390/pathogens11050609.
13. Kalra SK, Sharma P, Shyam K, Tejan N, Ghoshal U. *Acanthamoeba* and its pathogenic role in granulomatous amebic encephalitis. Experimental Parasitology. 2020; 208:107788. doi: 10.1016/j.exppara.2019.107788.
14. Qvarnstrom Y, Nerad TA, Visvesvara GS. Characterization of a new pathogenic *Acanthamoeba* species, *A. byersi* sp. isolated from a human with fatal amoebic encephalitis. Journal of Eukaryotic Microbiology. 2013; 60:626–633. doi: 10.1111/jeu.12069.
15. Khan NA, Jarroll EL, Panjwani N, Cao Z, Paget TA. Proteases as markers for differentiation of pathogenic and non-pathogenic *Acanthamoeba* strains. Journal of Clinical Microbiology. 2000; 38:2858–2861. doi: 10.1128/JCM.38.8.2858-2861.2000.
16. Costa AO, Castro EA, Ferreira GA, Furst C, Crozeta MA, Thomaz-Soccol V. Characterization of *Acanthamoeba* isolates from dust of a public hospital in Curitiba, Parana, Brazil. Journal of Eukaryotic Microbiology. 2010; 57:70–75. doi: 10.1111/j.1550-7408.2009.00453.x.
17. Khan NA, Jarroll EL, Paget TA. *Acanthamoeba* can be differentiated by the polymerase chain reaction and simple platting assays. Current Microbiology. 2001; 43:204–208. doi: 10.1007/s002840010288.
18. Basher MHA, Ithoi I, Mahmud R, Abdulsalam AM, Foead AI, Dawaki S, Atroosh WMM, Nissapatorn V, Abdullah WO. Occurrence of *Acanthamoeba* genotypes in Central West Malaysian environments. Acta Tropica. 2018; 178:219–228. doi: 101.1016/j.actatropica.2017.11.015.
19. Init I, Lau YL, Arin Fadzlun A, Foead AI, Neilson RS, Nissapatorn V. Detection of free-living amoebae, *Acanthamoeba* and *Naegleria* in swimming pools,

Malaysia. Tropical Biomedicine. 2010; 27:566–577.
20. Mohd Hussain RH, Abdul Ghani MK, Khan NA, Siddiqui R, Anuar TS. *Acanthamoeba* species isolated from marine water in Malaysia exhibit distinct genotypes and variable physiological properties. Journal of Water and Health. 2022; 20:54–67. doi: 10.2166/wh.2021.128.
21. Mohd Hussain RH, Ishak AR, Abdul Ghani MK, Ahmed Khan N, Siddiqui R, Shahrul Anuar T. Occurrence and molecular characterization of *Acanthamoeba* isolated from recreational hot springs in Malaysia: evidence of pathogenic potential. Journal of Water and Health. 2019; 17:813–825. doi: 10.2166/wh.2019.214.
22. Page F. A new key to freshwater and soil gymnamoebae: With instructions for culture. Freshwater Biological Association, Ambleside. 1988.
23. Schroeder JM, Booton GC, Hay J, Niszl IA, Seal DV, Markus MB, Fuerst PA, Byers TJ. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba* from humans with keratitis and from sewage sludge. Journal of Clinical Microbiology. 2001; 39:1903–1911. doi: 10.1128/JCM.39.5.1903-1911.2001.
24. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution. 2013; 30:2725–2729. doi: 10.1093/molbev/mst/197.
25. Landell MF, Salton J, Caumo K, Broetto L, Rott MB. Isolation and genotyping of free-living environmental isolates of *Acanthamoeba* spp. form bromeliads in Southern Brazil. Experimental Parasitology. 2013; 134:290–294. doi: 10.1016/j.exppara.2013.03.028.
26. Fewtrell L, Kay D. Recreational water and infection: a review of recent findings. Current Environmental Health Reports. 2015; 2:85–94. doi: 10.1007/s40572-014-0036-6.
27. Putaporntip C, Kuamsab N, Nuprasert W, Rojrung R, Pattanawong U, Tia T, Yanmanee S, Jongwutiwes S. Analysis of *Acanthamoeba* genotypes from public freshwater sources in Thailand reveals a new genotype, T23 *Acanthamoeba* bangkokensis sp. nov. Scientific Reports. 2021; 11:17290. doi: 10.1038/s41598-021-96690-0.
28. Salazar-Ardiles C, Perez-Arancibia A, Asserella-Rebollo L, Gomez-Silv B. Presence of free-living *Acanthamoeba* in Loa and Salado Rivers, Atacama Desert, Northern Chile. Microorganisms. 2022; 10:2315. doi: 10.3390/microorganisms10122315.
29. Kang H, Sohn HJ, Seo GE, Seong GS, Ham AJ, Park AY, Jung SY, Lee SE, Cho SH, Shin HJ. Molecular detection of free-living amoebae from Namhangang (southern Han River) in Korea. Scientific Reports. 2020; 10:335. doi: 10.1038/s41598-019-57347-1.
30. Salehi M, Spotin A, Hajizadeh F, Soleimani F, Shokri A. Molecular characterization of *Acanthamoeba* spp. from different sources in Gonabad, Razavi Khorasan, Iran. Gene Reports. 2022; 27:101573. doi: 10.1016/j.genrep.2022.101573.
31. Chan LL, Toh HC, Jaikwang K, Loo EM, Wong JH, Liew YK, Ong KH, Ooi SS. Report of rare genotypes of *Acanthamoeba* from soil source of the Payeh Maga Highland forest, North-eastern Sarawak, Malaysia. Acta Tropica. 2022; 229:106372. doi: 10.1016/j.actatropica.2022.106372.
32. Fuerst PA, Booton GC. Species, sequence types and alleles: Dissecting genetic variation in *Acanthamoeba*. Pathogens. 2020; 9:534. doi: 10.3390/pathogens9070534.
33. Mohd Hussain RH, Mohamad Isa NS, Kamaruddin KA, Abdul Ghani MK, Khan NA, Siddiqui R, Anuar TS. Morphological and molecular characterization of *Acanthamoeba* isolated from contact lens paraphernalia in Malaysia: highlighting the pathogenic potential of T4 genotype. Asian Pacific Journal of Tropical Medicine. 2020; 13:542–549. doi: 10.4103/1995-7645.296722.
34. Huang SW, Hsu BM. Isolation and identification of *Acanthamoeba* from Taiwan spring recreation areas using culture enrichment combined with PCR. Acta Tropica. 2010; 115:282–287. doi: 10.1016/j.actatropica.2010.04.012.
35. Hsu TK, Chen JS, Tsai HC, Tao CW, Yang YY, Tseng YC, Kuo YJ, Ji D Der, Rathod J, Hsu BM. Efficient nested-PCR-based method development for detection and genotype identification of *Acanthamoeba* from a small volume of aquatic environmental sample. Scientific Reports. 2021; 11:21740. doi: 10.1038/s41598-021-00968-2.
36. Richard RL, Ithoi I, Majid MA, Wan Sulaiman WY, Tan TC, Nissapatorn V, Lim YA. Monitoring of waterborne parasites in two drinking water treatment plants: A study in Sarawak, Malaysia. International Journal of Environmental Research and Public Health. 2016; 13:641. doi: 10.3390/ijerph13070641.
37. Possamai CO, Loss AC, Costa AO, Falqueto A, Furst C. *Acanthamoeba* of three morphological groups and distinct genotypes exhibit variable and weakly inter-related physiological properties. Parasitology Research. 2018; 117:1389–1400. doi: 10.1007/s00436-018-5824-8.
38. Corsaro D. On the diversity and clinical importance of *Acanthamoeba* spp. from Group 1. Parasitology Research. 2021; 120:2057–2064. doi: 10.1007/s00436-021-07171-2.
39. Tanveer T, Hameed A, Muazzam AG, Jung SY, Gul A, Matin A. Isolation and molecular characterization of potentially pathogenic *Acanthamoeba* genotypes from diverse water resources including household drinking water from Khyber Pakhtunkhwa, Pakistan. Parasitology Research. 2013; 112:2925–2932. doi: 10.1007/s00436-013-3465-5.

40. Hounkong K, Sornying P, Saechan V, Sawangjaroen N. Isolation and identification of *Acanthamoeba* spp from water and soil in Southern Thailand. Southeast Asian Journal of Tropical Medicine and Public Health. 2022; 53:9–20.
41. Hajialilo E, Behnia M, Tarighi F, Niyyati M, Rezaeian M. Isolation and genotyping of *Acanthamoeba* strains (T4, T9, and T11) from amoebic keratitis patients in Iran. Parasitology Research. 2016; 115:3147–3151. doi: 10.1007/s00436-016-5072-8.
42. Chan LL, Mak JW, Low YT, Koh TT, Ithoi I, Mohamed SM. Isolation and characterization of *Acanthamoeba* spp. from air-conditioners in Kuala Lumpur, Malaysia. Acta Tropica. 2011; 117:23–30. doi: 10.1016/j.actatropica.2010.09.004.
43. Nielsen MK, Nielsen K, Hjortdal J, Sorensen UB. Temperature limitation may explain the containment of the trophozoites in the cornea during *Acanthamoeba* castellanii keratitis. Parasitology Research. 2014; 113:4349–4353. doi: 10.1007/s00436-014-4109-0.
44. Booton GC, Rogerson A, Bonilla TD, Seal DV, Kelly DJ, Beattie TK, Tomlinson A, Lares-Villa F, Fuerst PA, Byers TJ. Molecular and physiological evaluation of subtropical environmental isolates of *Acanthamoeba* spp., causal agent of *Acanthamoeba* keratitis. Journal of Eukaryotic Microbiology. 2004; 51:192–200. doi: 10.1111/j.1550-7408.2004.tb00545.x.
45. Khan NA. *Acanthamoeba*, biology and pathogenesis (1st edition). Caister Academic Press. 2009.
46. Perez-Serrano J, Martinez J, Perez B, Bernadina WE, Rodriguez-Caabeiro F. In vitro shock response to different stressors in free living and pathogenic *Acanthamoeba*. International Journal for Parasitology. 2000; 30:829–835. doi: 10.1016/s0020-7519(00)00060-6.
47. Abdul Halim R, Mohd Hussain RH, Aazmi S, Halim H, Ahmed Khan N, Siddiqui R, Shahrul Anuar T. Molecular characterisation and potential pathogenicity analysis of *Acanthamoeba* isolated from recreational lakes in Peninsular Malaysia. Journal of Water and Health. 2023; 21: 1342–1356. doi:10.2166/wh.2023.186.
48. Corsaro D. Exploring LSU and ITS rDNA sequences for *Acanthamoeba* identification and phylogeny. Microorganisms. 2022; 10:1776. doi: 10.3390/microorganisms10091776.
49. Castro-Artavia E, Retana-Moreira L, Lorenzo-Morales J, Abrahams-Sandi E. Potentially pathogenic *Acanthamoeba* genotype T4 isolated from dental units and emergency combination showers. Memorias Do Instituto Oswaldo Cruz. 2017; 112: 817–821. doi: 10.1590/0074-02760170147.
50. Nuprasert W, Putaporntip C, Pariyakanok L, Jongwutiwes S. Identification of a novel T17 genotype of *Acanthamoeba* from environmental isolates and T10 genotype causing keratitis in Thailand. Journal of Clinical Microbiology. 2010; 48: 4636–4640. doi: 10.1128/JCM.01090-10.