PATHOGENIC *ACANTHAMOEBA GRIFFINI* GENOTYPE T3 ISOLATED FROM A GEOTHERMAL WATER PARK, MALAYSIA

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Abstract. Free-living amoebae of the genus *Acanthamoeba* can cause amoebic encephalitis and keratitis infections. Investigations have been conducted to identify the direct and indirect virulence parameters to establish the pathogenic potential of this amphizoic amoeba due to its widespread distribution. The current study sampled ten sites along a stream in Sungai Klah Geothermal Park, Perak, Malaysia, and seven sites showed the presence of *Acanthamoeba*. Using a combination of morphology examination and molecular analysis, the samples were identified as *A. castellanii, A. griffini, A. lenticulate* (putative), and *A. polyphaga*, belonging to genotypes T3 (n = 2), T4 (n = 4) and T5 (n = 1). An isolate SKGP-5, *A. griffini* genotype T3 exhibited the highest level of cytotoxicity (based on a HeLa cell monolayer assay and detection of secreted serine proteases) and potential pathogenicity as evidenced by tolerance to the temperature of 42°C and 1M mannitol (hyperosmotic solution). This is the first discovery of a pathogenic *A. griffini* genotype T3 isolate at a public recreational hot spring in Malaysia, indicating the need for regular monitoring of microorganisms that pose a threat to public health in such settings.

Keywords: *Acanthamoeba*, cytotoxicity, genotype, HeLa cell line, hot spring, pathogenicity

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INTRODUCTION

Free-living amoebae of the genus *Acanthamoeba* are widely distributed in marine systems, inhabiting natural and anthropogenic settings, such as freshwater bodies, hot springs, lakes, ponds, swimming pools, and tap water systems (de Lacerda and Lira, 2021). *Acanthamoeba* possesses remarkable adaptability, evidenced by its ability to thrive across diverse water types characterized by conditions posing significant challenges to numerous organisms, such as temperature fluctuation, high temperature and varying chemical composition.

Acanthamoeba has a complex life cycle involving trophic and cystic phases. In harsh conditions, *Acanthamoeba* trophozoites transform into resilient cysts that can remain viable for extended periods, surpassing 20 years (Sriram *et al*, 2008). Based on cyst morphology, *Acanthamoeba* is categorized into three groups (I, II and III) consisting of more than 25 nominal species (Booton *et al*, 2005). However, molecular and biochemical data indicate discrepancies in this classification (Corsaro and Venditti, 2010). Based on 18S rRNA sequences, the genus *Acanthamoeba* can be divided into 23 genotypes, namely T1 to T23 (Putaporntip *et al*, 2021).

Acanthamoeba genotype T4 is known for its pathogenic potential and environmental and clinical specimens are implicated in various diseases (Booton *et al*, 2005). Other genotypes, *viz* T2, T3, T5, T6, T10, T11, T12, T15, and T18, have sporadically been detected in clinical settings with potential pathogenicity (Possamai *et al*, 2018). *Acanthamoeba* is a primary causative agent of amoebic keratitis (AK), which predominantly affects contact lens users, but also occurs in nonusers. *Acanthamoeba* spp identified as causing keratitis

are *A. castellanii*, *A. culbertsoni*, *A. griffini*, *A. hatchetti*, *A. lugdunensis*, *A. polyphaga*, *A. quina*, and *A. rhysodes* (Schaumberg *et al*, 1998). *Acanthamoeba* has also been reported to induce chronic granulomatous amoebic encephalitis (GAE), primarily in immunocompromised individuals (Marciano-Cabral and Cabral, 2003). Some *Acanthamoeba* spp can cause cutaneous lesions, osteomyelitis, pulmonary infections, and rhinosinusitis (Cammaroto *et al*, 2015; Steinberg *et al*, 2002). However, despite recent advances, a complete understanding of *Acanthamoeba* pathogenicity is still lacking (Walochnik and Duchêne, 2016). *Acanthamoeba* pathogenicity is independent of genotypes but is influenced by such characteristics as growth rate, shape and adaptation to various host environments. Consequently, it is prudent to consider all isolates as potentially pathogenic unless shown otherwise (Khan *et al*, 2002).

Pathogenicity can be defined by phenotypic traits, such as osmo- and thermo-tolerance, production of secretory proteases and ability to induce damage to human cell lines (Khan, 2006). However, no universally applicable methods are available to establish definitively the latent pathogenicity of *Acanthamoeba*. Nevertheless, several properties have been identified that permit differentiation between infectious and non-infectious isolates. Paknejad *et al* (2020) suggest that *Acanthamoeba* osmo- and thermo-tolerance are sufficient for determining pathogenicity. Osmo-tolerance refers to the ability of amoebae to withstand high osmotic conditions encountered during corneal epithelium invasion and in tear fluid, and thermo-tolerance refers to survival at the host elevated temperatures.

Acanthamoeba extracellular proteases are critical in several disease processes, such as tissue infiltration and induction of host cell death (Alvarado-Ocampo *et al*, 2020). In the initial stages of AK, adherence via glycoproteins and lectins on the host cell surface is followed by protease(s) release that induces the cytopathic effects (CPE) (Clarke and Niederkorn, 2006). In GAE, secreted proteases also

contribute to the degradation of the blood-brain barrier (Alsam *et al*, 2005). *Acanthamoeba* produces serine, cysteine and metalloproteases, with distinct compositions (Khan, 2006). Biochemical analysis of the proteases has revealed their roles in host tissue penetration and cell lysis (Omaña-Molina *et al*, 2013). Moreover, assays assessing cell viability and cytotoxicity are valuable parameters in determining the effects of *Acanthamoeba* on human cells, including detecting structural alterations, membrane damage, and physiological and biochemical properties indicative of cell viability (Mbouaka *et al*, 2023).

Acanthamoeba cysts have remarkable adaptability. Cysts can detect environmental alterations, undergoing excystment under favorable conditions, namely optimal temperature (30-32°C) and osmolarity (50-80 mOsmol) (Garajová *et al*, 2019). Cysts with higher pathogenic potential can endure extreme conditions, potentially increasing *Acanthamoeba* infection risks, particularly in immunocompromised individuals. *Acanthamoeba* cysts are also considerably resistant to chemical treatments, posing challenges to water sanitation treatments of natural sources (Nageeb *et al*, 2022).

In Malaysia, geothermal water from hot springs holds significant cultural and recreational value. The hot springs are natural tourist attractions and are regarded as traditional therapeutic sites being rich in minerals, such as sulfur, radium and carbon dioxide, and providing therapeutic properties, such as improving blood circulation, relieving fatigue and muscle soreness, and enhancing skin health (Yazdi *et al*, 2015). Furthermore, popular destinations such as Sungai Klah Geothermal Park, Sungkai, Perak, with their distinct variations in temperature, mineral content and water flow rate also provide various amenities, such as swimming pools and spas, to cater to leisure activities (Simon *et al*, 2019).

The current study focused on identifying and genotyping *Acanthamoeba* specimens from a Malaysian geothermal leisure site. A comprehensive characterization of the pathogenicity properties

of the isolates was undertaken, resulting for the first time the identification of a pathogenic *A. griffini* T3 genotype from a Malaysian hot spring leisure site, highlighting the need for regular surveys of pathogenic microorganisms in public geothermal water parks.

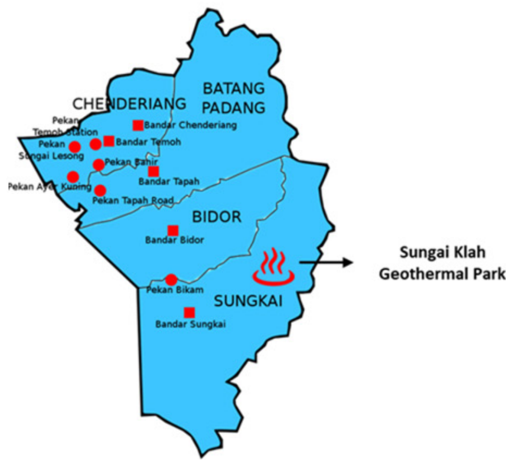
MATERIALS AND METHODS

Water sample collection and *Acanthamoeba* culture

Sungai Klah is adjacent to Sungkai, Perak, about 130 km from Kuala Lumpur. The Sungai Klah Geothermal Park is approximately 150 m in length and located (03° 59′ 40′′ N and 101° 23′ 33′′ E) at a principal fault line in the Main Range (Titiwangsa Mountain) (Fig 1), which consists of granite, sedimentary rocks and alkali feldspar (William *et al*, 2022). The Park is among the most effectively operated public hot springs in Malaysia.

A 1 l aliquot of water samples were collected from 10 locations along the Sungai Klah Geothermal Park stream at a depth of 10-20 cm between August and September 2018. The water temperature and pH of each sample were measured at the site using a thermometer and portable pH meter (HI9829; Hanna Instruments, Woonsocket, RI). Water samples were placed into sterile borosilicate Schott bottles (Schott AG, Mainz, Germany) and transported at 4°C within 24 hours to the Centre for Medical Laboratory Technology Studies, Universiti Teknologi MARA, Malaysia, where subsequent analyses were performed following the Public Health Association (APHA) and the United States Environmental Protection Agency (USEPA) guidelines (Milanez *et al*, 2020).

Each water sample was filtered through a cellulose nitrate membrane filter (0.45 μm pore size) (Sartorius, Gottingen, Germany). Then, each membrane was cut into four pieces and placed on 1.5% non-nutrient agar (NNA) plate (Sigma Aldrich A7002, St Louis, MO) containing a lawn of UV-inactivated Escherichia coli K12 (ATCC 10798) in Page's amoeba saline (PAS) solution pH 6.9 and



Sungai Klah Geothermal Park located at Sungkai, Perak, Malaysia

Sampling Site at Sungai Klah Geothermal Park

Fig 1 - Map of Sungai Klah Geothermal Park location and study sampling sites, Sungkai, Perak, Malaysia

incubated at 30°C at 85% relative humidity for up to two weeks (Init *et al*, 2010).

Microscopic assessment and culture establishment

Culture plates containing non-nutrient agar (NNA) (Sigma Aldrich, St Louis, MO) and *E. coli* lawn were incubated at 30°C, and examined daily for amoebic growth using an inverted light microscope (Leica DMI3000 B, Wetzlar, Germany) for two weeks before being declared positive or negative. A sample culture lasting two weeks is the gold standard for diagnosis. Standard *Acanthamoeba* trophozoite and cyst morphology was based on the appearance under a microscope (ZEISS Primo Star, Oberkochen, Germany), 1,000× magnification, of 10-25 μm spike-like pseudopodia (‘acanthopodia’) and spherical or wrinkled double wall respectively (Page, 1988). *Acanthamoeba* isolates were classified into three groups (I, II and III) based on cyst size and shape (Pussard and Pons, 1977).

Plates containing positive cultures were then sub-cultured 10 times. Colonies consisting of four to six cysts were added to freshly prepared *E. coli* in NNA to obtain homogeneous cultures. Trophozoites from each isolate were grown in five plates to obtain 5×10^5 (500,000) number of trophozoite cells.

Scanning electron microscopy assay

Trophozoites and cysts were treated with 4% glutaraldehyde for 2 hours at 4°C, washed three times with 0.1M phosphate buffer pH 7.2, treated with 1% osmium tetroxide for 2 hours at 4°C, washed twice with above buffer at 4°C, and dehydrated using increasing acetone concentrations (35, 50, 75, 95, and 100%) for 15 minutes at 4°C each concentration. Samples were then subjected to critical point drying, mounted on aluminum stubs for platinum sputter coating of ~35 nm thickness, and observed using a Quanta™ 450 FEG scanning electron microscope (Thermo Fisher Scientific, Hillsboro, OR) (2,500-10,000x magnification) (Behera and Satpathy, 2017).

DNA extraction, PCR protocol and amplicon sequencing

Each positive agar culture plate was submerged in 1 ml of PAS solution; amoebae were gently detached; the solution was centrifuged at 700g for 10 minutes and supernatant subjected to DNA extraction employing a QIAamp® DNA mini kit (Qiagen, Hilden, Germany) according the manufacturer's protocol. DNA yield and purity of the samples were assessed with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE) and stored at -20°C until used.

Acanthamoeba 18S rRNA gene region was amplified using genus-specific primers JDP1 (5' GGCCCAGATCGTTTACCGTGAA 3') and JDP2 (5' TCTCA-CAAGCTGCTAGGGGAGTCA 3') (Schroeder *et al*, 2001). PCR was carried out in a thermocycler (T100, Bio-Rad, Hercules, CA) as follows: 94°C for 3 minutes; 35 cycles of 94°C for 30 seconds, 57°C for 60 seconds, and 72°C for 60 seconds; with a final step of 72°C for 10 minutes. Each PCR

experiment included a positive control (*A. castellanii* ATCC 50492) and a negative (distilled water) DNA sample. The assay was performed in triplicate. *Acanthamoeba* 450 base pair (bp) amplicon was separated by 1.5% agarose gel-electrophoresis, stained with ethidium bromide (0.5 µg/ml for 10 min) and gel-excised band sent for sequencing (ABIPRISM BigDye® Terminator v.3.1 Cycle Sequencing Kit, Thermo Fisher Scientific, Waltham, MA).

Phylogenetic analysis

Editing and aligning the sequences obtained were conducted with the MEGA 6.0 software program (Tamura *et al*, 2013). Isolates' comparison and classification into different species were achieved with the Basic Local Alignment Search Tool program of the US National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). The nucleotide sequences of all *Acanthamoeba* genotypes were also obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/pubmed). Kimura-two-parameter distance algorithm utilizing 1,000 replicates and the neighbor-joining technique were employed to construct a phylogenetic tree. The final phylogenetic tree was rooted with the *Hartmanella* sp (NCBI KF697197) sequence.

Pathogenicity characterization

Two assays were employed to determine the pathogenicity of the *Acanthamoeba* samples, namely thermo- and osmo-tolerance assays. For the former assay, an NNA block containing *Acanthamoeba* cysts was placed in a culture plate and incubated at 37°C for a week and another block was incubated in parallel at 42°C. Amoeba growth on each plate was examined daily under a light microscope (ZEISS) (400× magnification). The experiment was performed in triplicate. For the latter assay conducted in this study, an NNA block containing *Acanthamoeba* cysts was cut into small pieces and placed in a culture plate, and overlaid with a freshly prepared solution of 1×10^6 *Escherichia coli*/ml 1.5% NNA in 0.5M mannitol and another sample was similarly prepared but containing 1.0M mannitol.

A non-mannitol-containing sample was used as a negative control. The plates were incubated for 7 days at 30°C and amoeba growth was determined based on the numbers of trophozoites or cysts, with 0 number recorded as (-), 1-15 (+), 16–30 (++) and ≥31 (+++) (Caumo *et al*, 2009). Each experiment was performed in triplicate. Isolates exhibiting growth at 42°C and in 1.0M were defined as significantly pathogenic, while those with growth at 37°C and in 0.5M mannitol as less pathogenic (Niyyati *et al*, 2016).

Protease assay

The protease activity of *Acanthamoeba* isolates considered significantly pathogenic was determined using a zymogen assay (Alfieri *et al*, 2000). *Acanthamoeba* trophozoite lysate (30-40 µl, equivalent to 30 µg of protein) was subjected to 10% SDS-PAGE containing 500 µg/ml copolymerized gelatin. Then gels were soaked in 2.5% Triton X-100 for 60 minutes, incubated in a developing buffer (50 mM Tris-HCl pH 7.5 containing 10 mM $CaCl_2$) overnight at 37°C and then stained with Coomassie brilliant blue R-250 dye (Bio-Rad Lab, Hercules, CA). Following de-staining, gel patterns were documented using a Gel Analyzer 19.1 system (István Lázár, Budapest, Hungary) (Tawfeek *et al*, 2016). To determine the presence of serine protease activity, *Acanthamoeba* trophozoite lysates were treated with 1mM phenylmethylsulfonyl fluoride (PMSF) (Nacalai Tesque Inc, Kyoto, Japan) for 30 minutes prior to gel-electrophoresis assay.

Cytotoxicity assay

In vitro cytopathic effect (CPE) of *Acanthamoeba* isolates was performed as previously described (Castro-Artavia *et al*, 2017). In brief, Hela cells (ATCC CCL-2) ($2×10^5$ cells/ml) were grown in Dulbecco's modified Eagle medium (DMEM) (HiMedia Lab Pvt Ltd, Maharashtra, India) supplemented with 10% (v/v) fetal bovine serum (Capricorn Scientific, Ebsdorfergrund, Germany), penicillin (100 U/ml) and streptomycin (100 pg/ml) for 24 hours at 37°C in a 24-well plate (NEST, Woodbridge, NJ) under an atmosphere of 5% CO_2, to attain confluency. *Acanthamoeba*

trophozoites (1×10^5) were added to each well; the control was a well without the addition of *Acanthamoeba* trophozoites. Following an hour of incubation, the unbound amoebae were eliminated by washing with 1X PBS, and the number of bound amoebae was determined by counting them with a hemocytometer. After 24 hours of incubation as described above, crystal violet was added to the wells, and cells were analyzed using ImageJ software (https://imagej.net/ij/download.html) (National Institutes of Health, Bethesda, MD). CPE of each sample was classified as follows: intact monolayer, (-); <10% damaged monolayer, (+); 10-50%, (++); and >50-100%, (+++) (Possamai *et al*, 2018). *A. castellanii* (ATCC 50492) was employed as a positive control.

In addition, supernatants were collected and lactate dehydrogenase (LDH) levels were measured using a Cytotoxicity Detection Kit (Roche Applied, Welwyn, UK). Percent cytotoxicity was calculated according to the following formula:

$$\text{Percent cytotoxicity} = (\text{Sample}_{A490\ nm} - \text{Control}_{A490\ nm})/(\text{Total}_{A490\ nm} - \text{Control}_{A490\ nm}) \times 100$$

where Control = HeLa cells incubated in DMEM,

Total = HeLa cells treated with 2% Triton X-100 at 37°C for 1 hour

Cytotoxicity is defined as follows: <10%, no; 10-25%, low; >25-40%, intermediate; and >40%, considerable (Lorenzo-Morales *et al*, 2010).

RESULTS

Acanthamoeba collected at Sungai Klah Geothermal Park

Based on the culture and direct microscopy results, 7/10 samples obtained from Sungai Klah Geothermal Park were identified as positive for *Acanthamoeba* (Table 1). The *Acanthamoeba* samples collected by the culture method were viable and generated visible colonies within a week.

Table 1

Presence of *Acanthamoeba* in ten sampling sites at Sungai Klah Geothermal Park, Sungkai, Perak, Malaysia

Sampling site	Latitude DMS	Longitude DMS	Culture-confirmed
SKGP-1	3° 59' 52.53'' N	101° 23' 36.34'' E	Positive
SKGP-2	3° 59' 48.31'' N	101° 23' 30.13'' E	Negative
SKGP-3	3° 59' 43.76'' N	101° 23' 23.58'' E	Positive
SKGP-4	3° 59' 41.11'' N	101° 23' 19.05'' E	Positive
SKGP-5	3° 59' 38.78'' N	101° 23' 14.94'' E	Positive
SKGP-6	3° 59' 34.33'' N	101° 23' 18.16'' E	Positive
SKGP-7	3° 59' 30.14'' N	101° 23' 13.64'' E	Positive
SKGP-8	3° 59' 35.61'' N	101° 23' 21.14'' E	Negative
SKGP-9	3° 59' 56.56'' N	101° 23' 40.35'' E	Negative
SKGP-10	3° 59' 50.42'' N	101° 23' 32.21'' E	Positive

DMS: degrees, minutes, seconds; E: east; N: north

Acanthamoeba cysts were 13-20 µm in size, double-walled, and had wrinkled ectocysts and polygonal endocysts, indicative of group II morphology (Figs 2A and 2B). Trophozoites were 12-45 µm in size and exhibited a flat shape, spine-like morphology, the typical appearance of *Acanthamoeba* (Fig 2C).

Acanthamoeba molecular characterization and phylogeny

Cultured *Acanthamoeba*-positive samples were confirmed by PCR-based detection of genus-specific 18S rDNA 450 bp amplicon (not shown). Amplicons were sequenced, revealing 98-100% similarity with NCBI *Acanthamoeba* reference strains for all seven amplicons. The sequences were deposited with GenBank, accession nos. MH791002-791008.

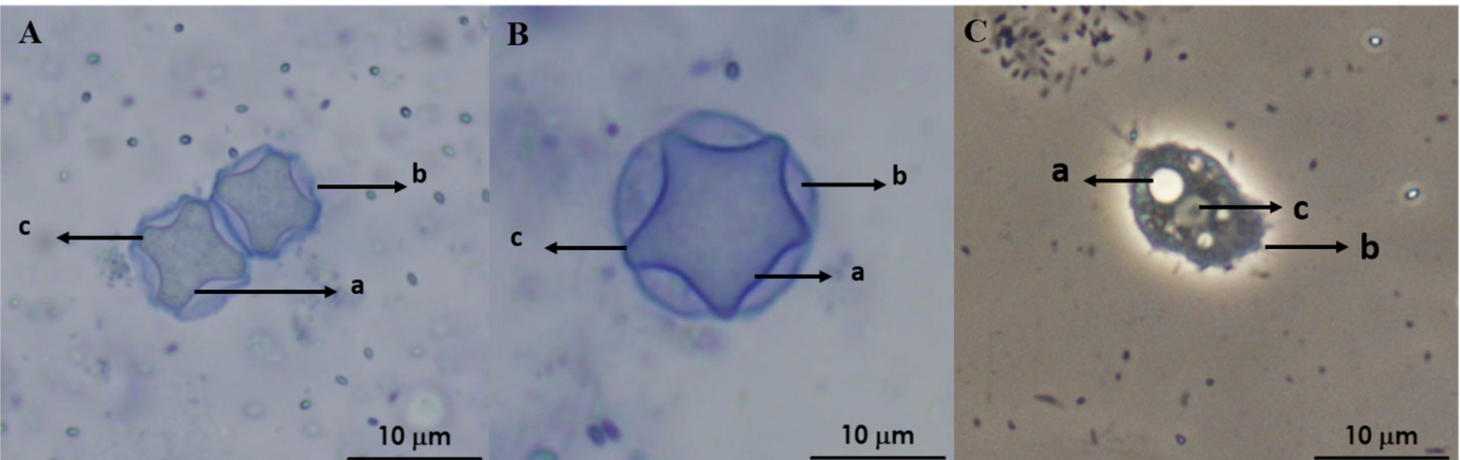
Neighbor-joining analysis based on the 450 bp 18S rDNA sequences and those of the reference strains deposited at NCBI GenBank showed that the *Acanthamoeba* isolates were identified as *A. castellanii* genotype T4 (n = 4), *A. griffini* genotype T3 (n = 1), *A. polyphaga* genotype T3 (n = 1), and an unidentified genotype T5 species (n = 1) (Fig 3). Thus, the *Acanthamoeba* isolates (except SKGP-7) collected from Sungai Klah Geothermal Park have the potential to cause AK (Schaumberg *et al*, 1998).

Acanthamoeba pathogenicity

Based on the results of thermo- and osmo-tolerance assays, all seven isolates exhibited thermo-tolerance at 37°C and six at 42°C; similarly, all isolates showed osmo-tolerance to 0.5M mannitol, but only isolate SKGP-5 (genotype T3) tolerated 1M mannitol (Table 2). The reference strain *A. castellanii* (ATCC 50492) (genotype T4) grew at 42°C and in 1M mannitol.

Protease secretion by the *Acanthamoeba* isolates

Zymography was employed to determine the number and type of proteases secreted by *Acanthamoeba* trophozoites (Fig 4). Isolate SKGP-1 (genotype T4) and SKGP-7 (genotype T5) secreted only one type of protease (124 and 130 kDa respectively), SKGP-4 and SKGP-10 (both genotype T4) two types (152+200 and 100+148 kDa



*Trophozoite: a – Contractile vacuole, b – Acanthopodia, c – Nucleus
*Cyst: a – Endocyst, b – Ectocyst, c – Ostiole

Fig 2 - Representative light microscope images (400× magnification) of *Acanthamoeba* predominant cyst morphotypes and trophozoite

A: Isolate SKGP-5 cysts; B: Isolate SKGP-7 cysts; C: Typical trophozoite

Note: *Acanthamoeba* isolates were cultured for five days in DMEM. SKGP-5 and SKGP-7 show Group II morphotype.

DMEM: Dulbecco's Modified Eagle Medium

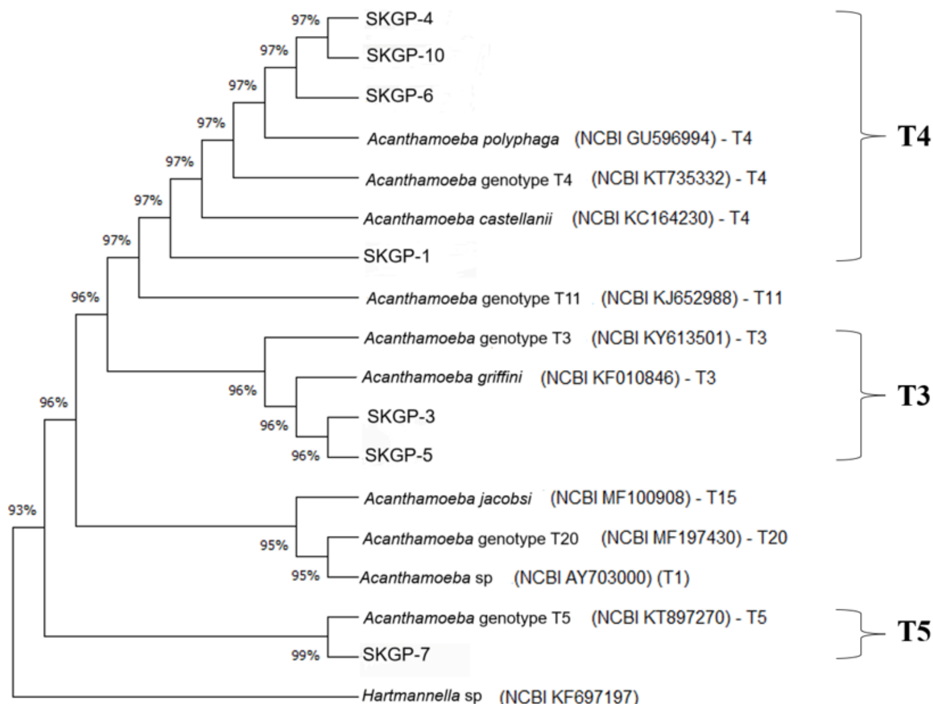


Fig 3 - Phylogenetic relationships among *Acanthamoeba* amplicons and reference isolates from NCBI GenBank

Note: The phylogenetic relationships were established through pairwise comparisons of 18S rRNA 450-bp amplicon sequences using neighbor-joining analysis, with *Hartmanella* sp (NCBI KF697197) as out-group.

bp: base pairs; NCBI: National Center for Biotechnology Information; rRNA: Ribosomal RNA

respectively), SKGP-3 (genotype T3) three types (56+75+148 kDa), SKGP-6 (genotype T4) four types (85+130+148+177 kDa), and SKGP-5 (genotype T3) five types (56+64+75+97+148 kDa). All secreted enzymes were serine proteases as evidenced by the absence of bands in the zymograms following treatment with the serine protease inhibitor PMSF (Fig 4). Of note, the 56 and 75 kDa serine proteases

Table 2

In vitro growth of *Acanthamoeba* isolates from water samples of Sungai Klah Geothermal Park, Sungkai, Perak, Malaysia

Sample identification	Genotype	Thermo-tolerance		Osmo-tolerance		Pathogenic potential
		37°C	42°C	0.5M mannitol	1M mannitol	
SKGP-1	T4	+++	-	++	-	Low
SKGP-3	T3	+++	++	++	-	Low
SKGP-4	T4	+++	-	++	-	Low
SKGP-5	T3	+++	+	+	+	High
SKGP-6	T4	+++	++	++	-	Low
SKGP-7	T5	+++	+	++	-	Low
SKGP-10	T4	+++	+	++	-	Low
Reference strain *A. castellanii* (ATCC 50492)	T4	+++	++	+++	++	High

Note: -, +, ++, and +++ indicates presence of 0, 1-15, 16-30, and >31 cysts and/or trophozoites, respectively.

M: Molar; °C: degree Celsius

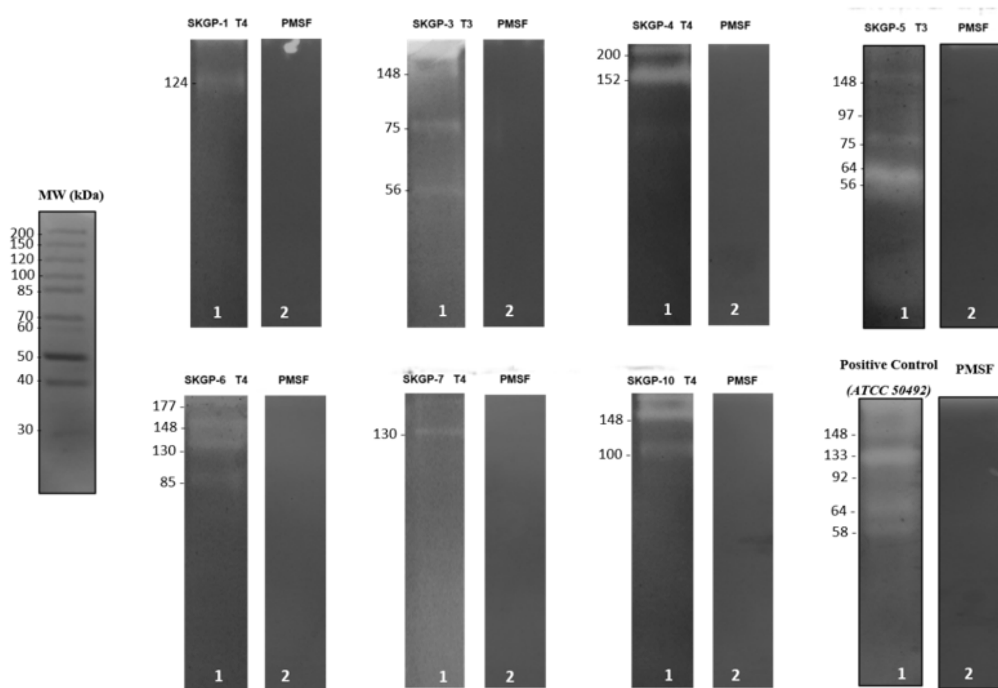


Fig 4 - Zymography analysis of *Acanthamoeba* trophozoite lysate from Sungai Klah Geothermal Park, Sungkai, Perak, Malaysia

Left panel - standard molecular weight markers

Right panel – Lane 1: Isolates without 1 mM phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor; Lane 2: Isolates pretreated with 1 mM phenylmethylsulfonyl fluoride (PMSF)

kDa: kilodalton; MW: molecular weight

were only secreted by genotype T3 *Acanthamoeba* trophozoites. The reference *A. castellanii* genotype T4 strain (ATCC 50492) secreted five types of serine proteases (58+64+92+133+148 kDa).

Cytotoxicity of *Acanthamoeba* isolates

The tested *Acanthamoeba* isolates were able to adhere to the HeLa cell monolayer with a percentage between 74.6-96.5%. Adhesion

assays revealed that *Acanthamoeba* genotype T3 (SKA5-SK35) isolates were able to adhere to the cell lines in a high percentage (>90%) (Fig 5). *In vitro* CPE of the *Acanthamoeba* isolates were evaluated using two methods, namely the extent of HeLa cell monolayer destruction and release of LDH. Damage to Hela cell monolayers was visualized by crystal violet staining following a 24-hour incubation with *Acanthamoeba* trophozoites (Fig 6). CPE of SKGP-5 isolate was highest (50%), followed by those of SKGP-1, -3, -6, and -7 isolates (13-39%), then those SKGP-4 and -10 isolates (≤10%). Positive control *A. castellanii* (ATCC 50492) produced the highest CPE (56%). Secreted LDH assay showed concomitant results: trophozoites of SKGP-5

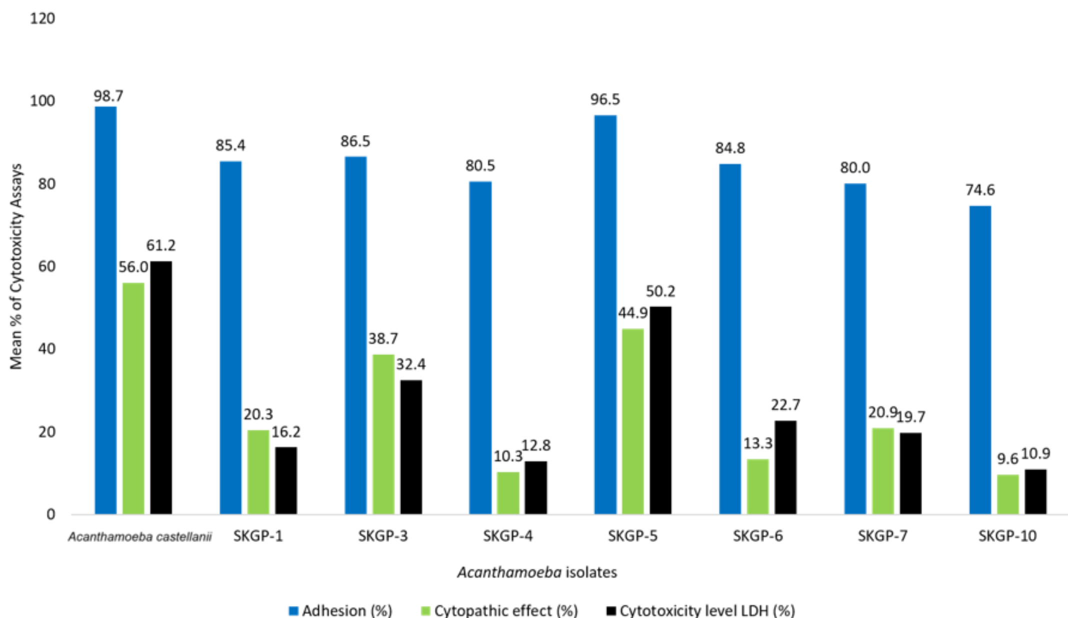


Fig 5 - Percent HeLa cells showing adhesion, cytopathic effect and cytotoxicity activity (LDH release) at 24 hours after inoculation with *Acanthamoeba* isolates

LDH: lactate dehydrogenase

isolate produced the highest level (50.2% of total activity from Hela cell lysate), comparable to that of control *A. castellanii* (61.2%) (Fig 6).

Morphology of *A. griffini* genotype T3 SKGP-5 isolate by scanning electron microscopy

Given that *A. griffini* genotype T3 SKGP-5 isolate exhibited properties

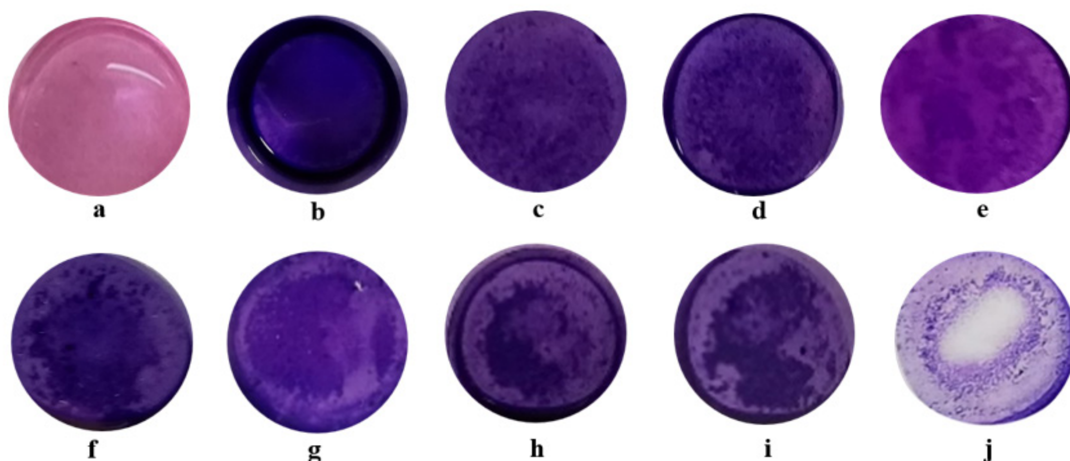


Fig 6 - Crystal violet staining of *Acanthamoeba* isolates grown on a monolayer of HeLa cells

Note: *Acanthamoeba* isolates were incubated with HeLa cells at 37°C for 24 hours and cytopathic effect (CPE) was determined using crystal violet staining.

a: cells grown in Dulbecco's Modified Eagle Medium without crystal violet staining; b: stained control cells; c: in presence of isolate SKGP-10; d: in presence of isolate SKGP-4; e: in presence of isolate SKGP-6; f: in presence of isolate SKGP-1; g: in presence of isolate SKGP-7; h: in presence of isolate SKGP-3; i: in presence of isolate SKGP-5; j: in presence of *A. castellanii* (ATCC 50492) (positive control).

Images are representative of experiments carried out in triplicate. CPE: ≤10% (c, d); 13-39% (e-h); 45% (i).

deemed considerably cytotoxic and potentially pathogenic, the morphologies of cysts and trophozoites were subjected to a more detailed examination using scanning electron microscopy (Fig 7). Trophozoites revealed rough and uneven cell surfaces with numerous projections; the varied-length acanthopodia occasionally divided into two branches; and flat and smooth lamellae were frequently observed (Figs 7A and 7B). The cyst hemisphere presented three to four opercula (pores), while the exocyst was generally spherical, with a wrinkled surface (Figs 7C-F). The electron microscope (EM) images provide ultrastructural details that cannot be seen with light microscopy and the images can be used as reference for further study that involves drug treatments or environmental stress conditions in which EM images can demonstrate changes in *Acanthamoeba* morphology as direct evidence of the study's findings.

DISCUSSION

Acanthamoeba is a ubiquitous free-living amoeba that thrives in diverse natural and artificial habitats, which underscores its exceptional adaptability (Siddiqui and Khan, 2012). Its ability to function as an opportunistic and non-opportunistic pathogen and its potential to harbor other microbes, such as bacteria and viruses, has made it a threat to public health (Fukumoto *et al*, 2016).

The current study sampled water from ten sites along a stream in Sungai Klah Geothermal Park to determine the presence of *Acanthamoeba* using culture, morphology and molecular techniques. *Acanthamoeba* was detected in 7/10 locations, in agreement with a previous report by Latiff *et al* (2018), which noted a 76% occurrence of this amoeba in several hot springs in Selangor, Malaysia. Similarly, studies in Iran reported the presence of *Acanthamoeba* in 50% of hot springs in the south-western, 47.8% in the northern and 20% in the north-western regions of the country (Solgi *et al*, 2012; Niyyati *et al*, 2016; Dodangeh *et al*, 2018)

The high contamination levels of *Acanthamoeba* in hot springs might

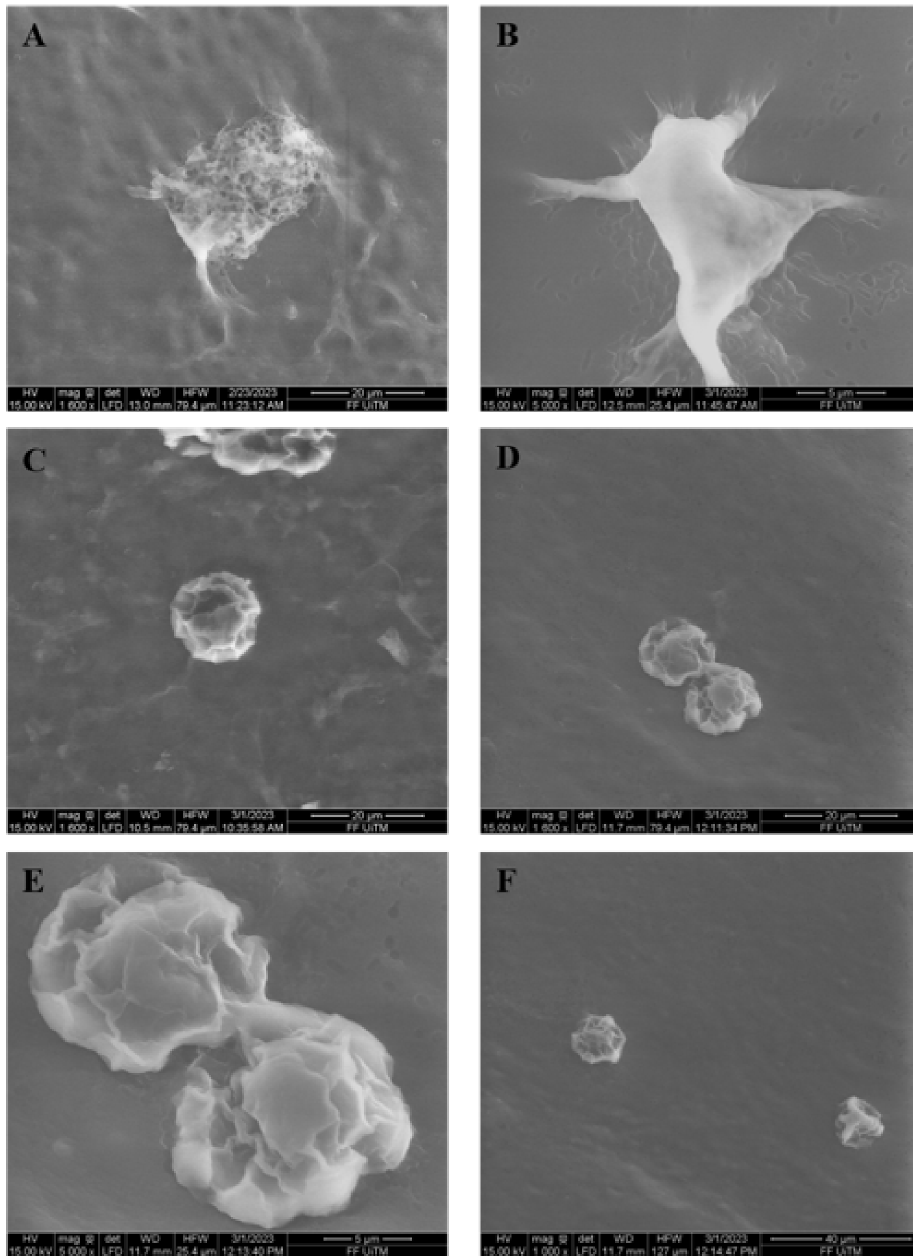


Fig 7 - Scanning electron photomicrographs of *Acanthamoeba griffini* T3 (isolate SKGP-5)

A and B: trophozoites; C-F: cysts

be attributed to the considerable number of visitors and natural contaminants promoting bacterial biofilm formation on geothermal tub walls. The biofilms attract *Acanthamoeba* that feed on various types of bacteria (Khan, 2006; Bagheri *et al*, 2010). In addition, Mohd Hussain *et al* (2019) noted a significant association between *Acanthamoeba* in geothermal waters and sulfate content, suggesting that sulfur-containing geothermal water creates a favorable environment for *Acanthamoeba* to thrive.

Comparisons of partial 18S rDNA sequences with those deposited with NCBI GenBank allowed the seven *Acanthamoeba* isolates to be identified as *A. castellanii* (genotype T4), *A. griffini* and *A. polyphaga* (both genotype T3), and an unidentified species with genotype T5, but suspected to be *A. lenticulate* (discussed later). All three *Acanthamoeba* spp. are capable of causing AK keratitis (Schaumberg *et al*, 1998)

Genotype T4 is commonly linked to AK cases (Kao *et al*, 2014) and was found in 4/7 samples from Sungai Klah Geothermal Park, thereby posing a potential public health risk. Solgi *et al* (2012) and Niyyati *et al* (2016) reported a high prevalence (83.3 and 93.7% respectively) of this genotype in Iran.

Genotype T3 was the second most prevalent genotype detected in this study. The latent pathogenicity of *A. griffini* genotype T3 is well-established, causing keratitis and less commonly GAE (Hewett *et al*, 2003). Pathogenic T3 isolates have also been reported in the UK (Ledee *et al*, 1996), central France (Risler *et al*, 2013) and Spain (Arnalich-Montiel *et al*, 2014).

Genotype T5 is also a cause of keratitis, as previously reported in the United States and Greece (Spanakos *et al*, 2006; Ledee *et al*, 2009). *A. lenticulata* (genotype T5) has been documented as a frequent contaminant in Taiwan recreational spring locations (Huang and Hsu, 2010). Acute granulomatous encephalitis in immunocompetent individuals (Lackner *et al*, 2010) and mucosal tissue infections in immunocompromised patients have also been linked to genotype

T5, potentially associating this genotype to ocular and brain pathologies (Javanmard *et al*, 2022).

Osmo- and thermo-tolerance are indicators associated with *Acanthamoeba* pathogenicity. The capacity of the amoebae to grow at temperatures exceeding 40°C is correlated with their ability to cause in vitro cellular damage, including CPE (Walochnik *et al*, 2000). The ability of *Acanthamoeba* to thrive in high salt (hyperosmotic) concentrations, such as tear fluid, and to adapt to physiological body temperature, are determinants of pathogenicity within *Acanthamoeba* genus (Booton *et al*, 2004). Among the seven *Acanthamoeba* isolates tested, only *A. griffini* SKGP-5 genotype T3 isolate demonstrated survival in 1M mannitol and at 42°C.

Twenty percent of hot therapeutic springs in north-western Iran contained *Acanthamoeba* isolates capable of growing above 40°C (Solgi *et al*, 2012). Furthermore, Rohr *et al* (1998) observed that *Acanthamoeba* isolates from hospital hot water systems are vulnerable to temperatures above 40°C, even though the parasite's capacity to survive at such high temperatures is uncommon. In a study of forty isolates of *Acanthamoeba* group III, Flint *et al* (2003) reported an *A. griffini* genotype T3 isolate that grows in 1M but not at 42°C. Kot *et al* (2018) suggested the ability of amoebae to thrive at 42°C is due to elevated expression levels of heat shock proteins HSP60 and HSP70. Other factors include the presence of an efficient osmo-regulation mechanism (Wang *et al*, 2023), membrane adaptation (Siddiqui and Khan, 2010) and biofilm formation (Marciano-Cabral and Cabral, 2003).

The current study confirmed that pathogenic *Acanthamoeba*e can adhere to HeLa cell monolayers (Alvarado-Ocampo *et al*, 2020) as evidenced by isolates belonging to genotypes T3, T4 and T5. In particular, *A. griffini* genotype T3 SKGP-5 isolate demonstrated 96.5% adhesion to HeLa cell monolayers, consistent with the report by Martin-Navarro *et al* (2010), which employed other types of cell lines.

González-Robles *et al* (2014) also documented successful *A. griffini* genotype T3 trophozoite attachment to Madin-Darby canine kidney cell monolayer. According to Alsam *et al* (2005), variations in HeLa cell adherence might be due to differences in mannose-binding proteins and contact-dependent mechanisms among various amoeba strains.

A. griffini genotype T3 SKGP-5 isolate exhibited an intermediate capability to degrade the HeLa cell monolayer compared to control A. castellanii. Attachment of *A. griffini* genotype T3 trophozoite to Madin-Darby canine kidney cell line also results in cell damage after 24 hours (González-Robles *et al*, 2014). Another cytotoxicity property was LDH secretion from damaged HeLa cells, which correlated with the observed CPE. Martín-Navarro *et al* (2010) also recorded 55-75% cytotoxicity levels for *Acanthamoeba* genotype T3 CLC-16 strain.

Among the factors that trigger CPE by *Acanthamoeba* are soluble secretory products, such as serine and cysteine proteases and hydrolytic enzymes (Panjwani, 2010). Identifying and characterizing the proteases are essential in unveiling their role in pathogenicity. All *Acanthamoeba* isolates evaluated in the current study secreted serine proteases ranging from 56 to 200 kDa in agreement with a previous report (Cirelli *et al*, 2020). *A. griffini* genotype T3 SKGP-5 isolate secreted the highest number (n = 5) of protease types and also produced the highest protease activity among the seven isolates assessed (not shown). Serrano-Luna *et al* (2006) reported that pathogenic *Acanthamoeba* demonstrated superior extracellular protease activity. Interestingly, the two *Acanthamoeba* genotype T3 isolates secreted a 56 kDa protease that was detected in an isolate from a contact lens-wearing keratitis patient in Spain (Heredero-Bermejo *et al*, 2015). The secreted protease(s) of *Acanthamoeba* may enhance *Acanthamoeba* evasion mechanisms from the immune system of its hosts, yielding persistent infections (Ilyas *et al*, 2024). Serine proteases secreted by *Acanthamoeba* trophozoites can

also suppress humoral immune responses by cleaving IgA and IgG antibodies, contributing to the ability of the amoeba to evade the human immune responses (Ilyas *et al*, 2024).

The current study highlighted *A. griffini* genotype T3 SKGP-5 isolate pathogenic potential. The *in vitro* cytotoxicity results also validated the considerable pathogenicity of the isolate, which was further supported by the morphological characteristics (uneven cell surfaces with numerous projections and acanthopodia, including some bifurcating branches) observed by scanning electron microscopy. *Acanthamoeba*e with over 100 acanthopodia per cell are considered more pathogenic than cells with 20 acanthopodia per cell due to their increased binding affinity to mannose- and laminin-binding proteins, thereby enhancing their adherence to host cells (Lorenzo-Morales *et al*, 2013; Huth *et al*, 2017).

The first report linking *A. griffini* to human disease involved an AK-causing strain isolated from a domestic water supply used to clean a contact lens case (Ledee *et al*, 1996). The finding underscores the public health implications of waterborne *Acanthamoeba*. Genotype T3 in clinical cases across various countries has also been documented, with prevalence between 1 and 13% in China, France, Hong Kong, Iran, Japan, Spain, Sweden, the UK, and the USA (González-Robles *et al*, 2014).

Although genotype T4 is responsible for approximately 80% of *Acanthamoeba* infections worldwide due to its abundance (Risler *et al*, 2013), genotypes T3 and T11 are also commonly associated with AK (Maciver *et al*, 2013). However, infections arising from non-T4 genotypes, are typically more aggressive with poorer clinical outcomes than their T4 counterpart (Diehl *et al*, 2021). Infections with non-T4 genotypes frequently result in delayed diagnosis, leading to complications, such as scleritis, fixed dilated pupils, intumescent cataracts, and retinal detachment (Arnalich-Montiel *et al*, 2014). Patients infected with genotypes T3 and T11 often exhibit poor treatment

response and visual prognosis, with a significant possibility of extra-corneal disease manifestation, and may require surgical intervention (Arnalich-Montiel *et al*, 2014).

In conclusion, the current study identified in a stream within a recreational geothermal public park in Malaysia the presence of four *Acanthamoeba* spp (*A. castellanii, A. griffini, A. lenticulate* (putative), and *A. polyphaga*) belonging to genotypes T3, T4 and T5. *A. griffini* genotype T3 SKGP-5 isolate was shown to be cytotoxic with potential pathogenic properties. *Acanthamoeba* spp of genotype T3 are known causal agents of amoebic keratitis, affecting predominantly contact lens users, but also nonusers. These findings underscore the importance of understanding the transmission and pathogenic mechanisms of *Acanthamoeba* existing in recreational water environments. The significant pathogenicity of genotype T3 requires extensive epidemiological and pathogenicity studies on a global scale to determine its role as a pathogen of significance in environmental and clinical situations.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

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