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Assessment of *in vitro* dynamics of pathogenic environmental *Acanthamoeba* T4 and T9 genotypes isolated from three recreational lakes in Klang Valley, Malaysia over the HaCaT cell monolayer

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ABSTRACT

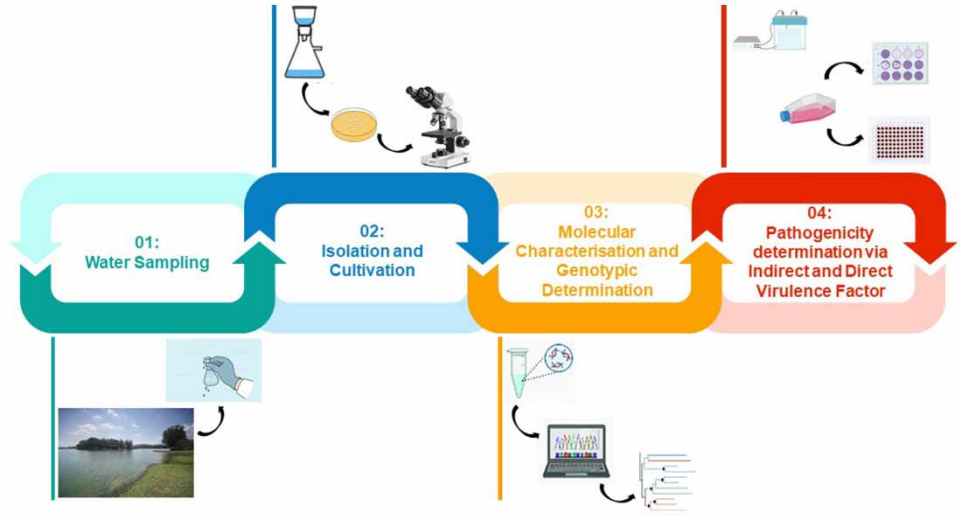
Free-living amoebae of the genus *Acanthamoeba* are causative agents of keratitis and amoebic encephalitis. They are widely found in various ecological environments. Therefore, the present study brings results that can help to better understand the genotypes of the environmental isolates and their pathogenicity. This study procured 26 *Acanthamoeba* isolates from three recreational lakes in 2022. Polymerase chain reaction amplification was performed on positive *Acanthamoeba* samples. The thermotolerance, osmotolerance, and cytopathogenicity in human keratinocyte (HaCaT) cells of the samples were also evaluated. The phylogenetic analysis demonstrated that 12 isolates were of genotype T4, two (T9), six (T17), four (T8), and one each from T5 and T11. The thermo- and osmotolerance assays indicated that eight *Acanthamoeba* samples were potentially pathogenic. Two T4 and one T9 genotype also recorded 33-, 42-, and 133-kDa serine-type proteases, respectively. The HaCaT cell monolayer revealed that three T4 and one T9 samples achieved cytopathic effects within the 50–100% range, hence significantly cytotoxic. The lactate dehydrogenase secretion results demonstrated that three (T4) and one (T9) sample exhibited exceptional toxicity (over 40%) compared to the other samples. The responses of *Acanthamoeba* members with similar genotypes to pathogenicity indicator assays varied considerably, rendering correlation of pathogenicity with specific genotypes challenging.

Key words: *Acanthamoeba*, cytopathic effect, HaCaT cells, Malaysia, pathogenicity, protease

HIGHLIGHTS

- This study showed first evidence of pathogenic *Acanthamoeba* T4 and T9 genotypes in recreational lakes in Malaysia.
- Two T4 and one T9 recorded 33-, 42-, and 133-kDa serine-type proteases, respectively.
- Three T4 and one T9 samples achieved cytopathic effects (50–100%) over the HaCaT cell monolayer.
- LDH secretion demonstrated three T4 and one T9 exhibited exceptional toxicity (over 40%).

GRAPHICAL ABSTRACT



INTRODUCTION

Acanthamoeba is a genus of aerobic protozoa thriving in numerous natural and artificial settings, such as fresh and tap water, soil, sand, dust, and sewage. The organism was also detected in healthy human nasal passages (Siddiqui & Khan 2012; Tawfeek *et al.* 2016). *Acanthamoeba* could survive as free-living protozoa or pathogens of vertebrates and hence are amphizoics (Fanselow *et al.* 2021). The species is among the most commonly isolated eukaryotic from the environment following the ability of its trophozoites to encyst, hence withstanding adverse surroundings (Alfieri *et al.* 2000). The *Acanthamoeba* have become relevant for public health, as they are natural hosts for numerous intracellular pathogens (Khan 2006). Furthermore, several species are correlated to two types of human illnesses: granulomatous amoebic encephalitis (GAE) and amoebic keratitis (AK) (Kot *et al.* 2018). The GAE is an opportunistic and often fatal infection affecting immunocompromised hosts. Conversely, AK affects healthy individuals, specifically contact lens wearers and might result in severe corneal damage (Cope *et al.* 2020).

Several *Acanthamoeba* species are classified according to their morphology and susceptibility to temperature (Pussard & Pons 1977). Nevertheless, not all the attributes are linked to human pathological occurrences. Although pathogenic potential determining factors are yet to be established entirely, several species such as *Acanthamoeba culbertsoni*, *Acanthamoeba castellanii*, *Acanthamoeba polyphaga*, *Acanthamoeba hatchetti*, and *Acanthamoeba healy* are more commonly detected in human infections (Schuster & Visvesvara 2004). Researchers internationally are employing a rapid and reliable detection approach based on the nuclear 18S small subunit ribosomal RNA gene to detect *Acanthamoeba* specimens (Maciver *et al.* 2013). Three remarkably informative *Rns* segments designated diagnostic fragments 1, 2, and 3 (DF1, DF2, and DF3) could yield robust phylogenetic trees, as they are based on the entire gene. Furthermore, the single significant variable and informative region, DF3 could allow prompt genotypic establishment (Kong 2009). As a typing criterion, strains under 5% difference in the 18S rRNA gene region are categorized as a single genotype (Siddiqui & Khan 2012). In total, 23 genotypes (T1–T23) have been identified via molecular characterization and phylogenetic analyses (Putaporntip *et al.* 2021). Some of the characterized genotypes are pathogenic, including T2, T3, T4, T5, T6, T11, and T15 with T4 as the most prevalent genotype isolated from human and environmental samples (Kalra *et al.* 2020). Identifying the genotypes of *Acanthamoeba* isolates from various ecological settings is essential for evaluating their virulence and tracking their epidemiological patterns, which can contribute to more effective public health measures.

Physiological and biochemical attributes and cytopathic effect (CPE) assays have allowed pathogenic and non-pathogenic *Acanthamoeba* differentiation (Mohd Hussain *et al.* 2022). Identifying pathogenic and non-pathogenic *Acanthamoeba* is critical for clinical diagnosis as the pathogenesis is correlated to numerous parameters. For instance, proteases are associated with host cell and tissue invasions and damage (Khan 2006). Pathogenic *Acanthamoeba* strains have higher temperature tolerance, growth rates and adherence characteristics, including greater cytotoxic product secretions and superior immune

evasion mechanisms, than their non-pathogenic counterparts (Marciano-Cabral & Cabral 2003). Although *Acanthamoeba* isolates predominantly produce serine proteases, they also secrete distinctly patterned cysteine and metalloproteases (Khan 2006). Nonetheless, clinical isolates produce more extracellular proteases than environmental samples (Kim *et al.* 2006; Lorenzo-Morales *et al.* 2015). Several studies reported identifying 107 and 133 kDa serine proteases, significant virulence factors in pathogenic *Acanthamoeba* (Khan *et al.* 2000; Huang *et al.* 2017). *Acanthamoeba* isolates with significant pathogenic attributes also reflect a temperature tolerance of over 42 °C and exhibit remarkable proteolytic enzyme activities (Kot *et al.* 2018).

The trophozoites of different *Acanthamoeba* species spontaneously release proteinases into culture media, such as a plasminogen activator, collagenolytic enzymes, cysteine proteinases and possibly elastase-like enzymes and metalloproteinases (Carvalho-Silva *et al.* 2021). The enzymes result from contact-independent mechanisms, which might also assist parasitic human tissue colonization's. Regulating *Acanthamoeba* in the surroundings is crucial following its opportunistic nature and possible role as a human pathogen reservoir. The present study aimed to characterize the inherent pathogenic attributes of the *Acanthamoeba* samples. Physiological (thermo- and osmotolerance) and biochemical (proteolytic activities) evaluations were performed on the *Acanthamoeba* isolated from recreational lakes to verify their pathogenic potential. This study also determined the genotypes of the possibly pathogenic environmental isolates to associate pathogenicity with specific genotypes. This focus is crucial, as prior research has revealed significant differences in pathogenicity.

MATERIALS AND METHODS

Isolation and cultivation of *Acanthamoeba* samples

The current study employed 26 monoxenic *Acanthamoeba* isolates. In total, 10 samples were procured from Biru Lake (N: 3.2475°, E: 101.5263°) and eight samples each from Titiwangsa (N: 3.1781°, E: 101.7065°) and Shah Alam (N: 3.0729°, E: 101.5138°) Lakes. Each sample was inoculated centrally on a non-nutrient agar (NNA) plate (Sigma Aldrich, St. Louis, MO, USA), containing Page's amoeba saline (PAS) solution at pH 6.9. The agar was overlaid with ultraviolet (UV)-inactivated *Escherichia coli* (*E. coli*) (strain K12, ATCC, Manassas, VA, USA). The NNA plates were incubated at 30 °C and were microscopically observed daily with an inverted microscope under ×100 magnification for up to 72 h (Mohd Hussain *et al.* 2022). The present study also procured cloned cultures via dilution (Costa *et al.* 2010). A single trophozoite or cyst was employed for the cloning procedure. Subsequently, the cultures were transferred to a 1.5% NNA plate before conducting physiological assessments. This study employed *A. castellanii* (ATCC 50492) as the positive control.

Extraction of DNA, amplification assay by polymerase chain reaction, and genotype isolation

The deoxyribonucleic acid (DNA) of the *Acanthamoeba* samples evaluated in the current study was extracted from its monoxenic culture with QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany). The methodology was applied following manufacturer guidelines. The DNA samples were stored at −20 °C until further use.

The polymerase chain reaction (PCR) assay genus-specific primer sets JDP1 and JDP2 employed in the present study were designed for *Acanthamoeba* genotyping (Schroeder *et al.* 2001). These primers, with sequences 5′-GGCCCAGATCGTTTACCGTGAA-3′ and 5′-TCTCACAAGCTGCTAGGGGAGTCA-3′ were established to amplify a fragment of 423–551 base pairs (bp) from the 18S rRNA region specific to *Acanthamoeba*, known as the *Acanthameoba*-specific (ASA.S1) amplimer. Each reaction was performed in triplicates with a final volume of 50 μL, consisting of 25 μL of TopTaq Master Mix (2×) (Qiagen, USA), 2 μL of each 10 μM of forward primers and 10 μM of reverse primers, 20 μL of DNase-free deionized water and 1 μL of DNA template (extracted DNA). The positive and negative controls in this study were prepared by incorporating the *A. castellanii* (ATCC 50492) DNA extract and DNase-free water (substituting the DNA template) into the reaction mix, respectively.

This study employed the GenJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) to purify the PCR products based on the protocol outlined by the manufacturer. Subsequently, a BigDye® Terminator v.3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) was utilized during forward and reverse sequencing. The resulting partial gene sequences of the 18S rRNA were subsequently analyzed using the Basic Local Alignment Search Tool (BLAST) program, hosted by the US National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nih.gov/BLAST) to categorize the *Acanthamoeba* isolates into distinct species. This analysis involved comparing the obtained sequence with all confirmed *Acanthamoeba* genotypes in the GenBank database, accessed through the 80 NCBI platform (https://www.ncbi.nlm.nih.gov/pubmed) to determine the closest matching sequence (Mohd Hussain *et al.* 2022). Multiple sequence alignment of all

Acanthamoeba-positive sequences obtained from this study, along with relevant reference sequences from GenBank was performed using ClustalW. These aligned sequences were then subjected to phylogenetic analysis using the MEGA software tool, v.11.0.13 (Mega Software, Tempe, Arizona, USA) (Tamura *et al.* 2013) with *Balamuthia mandrillaris* (NCBI KU184269) utilized as the outgroup. Phylogenetic trees were created based on the neighbour-joining distance tree method, which generated 1,000 bootstrapped replicates. The highest similarity percentages were recorded to establish the genotype and species.

Physiological assays

In this study, thermo- and osmotolerance 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 by Khan *et al.* (2001). Two culture plate sets were prepared for the assays.

In the thermotolerance 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 blocks 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 osmotolerance 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 ×400 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 (Landell *et al.* 2013). *A. castellanii* (ATCC 50492) was used as the reference strain to confirm pathogenic characteristics.

Preparation of *Acanthamoeba* trophozoite lysate

The trophozoites in this study were monitored daily for growth and collected after 3 days being subcultured on the NNA plates. The trophozoites were delicately procured from the agar surface of a minimum of three plates from each sample by adding 1–2 mL of sterile PAS solution. Gentamycin (100 g/mL) was also added upon washing the suspension twice with a cold PAS solution. Subsequently, the mixture was centrifuged at 3,500 rpm for 10 min.

The pellets obtained were lysed at 1.5×10^7–2.5×10^7 trophozoites/mL. The 0.15–0.20% (v/v) Triton® X-100 prepared in water with or without proteinase inhibitor was incorporated, followed by one or two cycles of rapid freezing and thawing. The protein contents of the pellets were determined based on the procedure reported by Tawfeek *et al.* (2016), with slight modifications. Protein standards were prepared using bovine serum albumin (BSA) as the reference. The Bradford reagent was then added to both the prepared standards and *Acanthamoeba* lysate samples. These mixtures were incubated for 5–10 min at room temperature to allow for dye-protein interaction. After incubation, the absorbance was measured at 595 nm using a spectrophotometer. Finally, the protein concentration of each sample was determined by comparing the absorbance values against a standard curve generated from the BSA standards.

Zymography assay for protease secretion determination and characterization

The current study utilized zymographic assays to determine the extracellular proteolytic reactions of the *Acanthamoeba* samples (Alfieri *et al.* 2000). *Acanthamoeba* trophozoite lysates were employed for the assessment. The zymography was prepared on 10% SDS-polyacrylamide gels copolymerized with 1% gelatin. The prepared sample buffer was mixed with the *Acanthamoeba* trophozoite lysate to yield a final volume of 30–40 μL (equivalent to 30 μg protein) before application on the gels.

A protein ladder of 10–250 kDa was used as the molecular size marker in the electrophoresis. Post-electrophoresis, the gels were soaked in 2.5% Triton® X-100 (w/v) solution for 60 min and incubated at 37 °C in a developing buffer (50 mM of Tris-HCl, pH 7.5, 10 mM of $CaCl_2$) overnight. Finally, the gels were rinsed and stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA). The electrophoresis gels were de-stained until clear, distinct, non-stained regions were observed against the dark blue background, indicating protease activities (Tawfeek *et al.* 2016). The de-staining process was terminated by applying a storage solution to the gels.

Protein molecular weights were determined using a gel documenting system software (Gel Analyzer 19.1). Similar experimental techniques were replicated on the trophozoite lysates from all samples. The isolate samples were pretreated with three protease inhibitors for 30 min before electrophoresis. In this study, 1 mM of phenylmethylsulphonyl fluoride

(PMSF), 20 mM of *N*-ethylmaleimide (NEM), and 10 mM of ethylenediaminetetraacetic acid (EDTA) were employed as serine proteases, cysteine proteases, and metalloproteases inhibitors, respectively (Omana-Molina *et al.* 2013).

Determination of the *in vitro* effect of *Acanthamoeba* on cell culture

HaCat (human keratinocyte) cell lines and culture conditions

The present study procured human keratinocyte (HaCaT) cells (CLS Cell Lines Service, 300493) from the Cell Line Service GmbH (Eppelheim, Germany). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (©Capricorn Scientific, Ebsdorfergrund, Germany), which consisted of penicillin (100 U/mL), streptomycin (100 pg/mL) (Hi-Media Laboratories Pvt. Ltd, Maharashtra, India), and 10% fetal bovine serum (©Capricorn Scientific, Ebsdorfergrund, Germany) (Mohd Hussain *et al.* 2022).

The HaCaT cells were preserved in a 5% carbon dioxide (CO_2) in air incubator at 37 °C and 80% humidity. After removing the existing media, 2 mL of trypsin was added to the confluent flasks (©Capricorn Scientific, Ebsdorfergrund, Germany) to detach the cells. The cells were resuspended in new media and incubated in 24-well plates (NEST®, Woodbridge, VA, USA). The samples were utilized in the cytotoxicity and cytopathogenicity assays after a homogenous monolayer was developed and verified microscopically within 48 h.

CPE assay

The HaCaT cell lines (CLS Cell Lines Service, 300493) cultured in this study were employed to determine the *in vitro* cytopathic effects (CPEs) of the *Acanthamoeba* samples (Mohd Hussain *et al.* 2022). The cells were grown in DMEM supplemented with penicillin (100 u/mL), streptomycin (100 pg/mL) (HiMedia Laboratories Pvt. Ltd, Maharashtra, India), and 10% fetal bovine serum (©Capricorn Scientific, Germany) at 37 °C and 5% CO_2 in air for 24 h. The current study experiments were in quadruplicates, with 5×10^5 *Acanthamoeba* trophozoites employed per well on 24-well plates (NEST®, USA). Each well contained a confluent cell monolayer with an amoebae-to-cell ratio of 1:2. The wells containing HaCaT cells that remained detached from the trophozoites were employed as control samples.

Crystal violet was utilized to stain the wells after being incubated for 24 h before performing a macroscopical assessment of visible alterations. In this study, the *in vitro* CPEs was analyzed based on the degree of monolayer cell damage by utilizing the ImageJ software. The results were categorized as no CPE (−), CPEs with up to 10% monolayer destruction (+), CPEs between 10 and 50% monolayer damage (++) and CPEs with 50–100% monolayer disruption (+++) (Possamai *et al.* 2018). The *A. castellanii* (ATCC 50492) strain was the positive CPE control in this study.

Host cell *in vitro* cytopathogenicity assay

The cytotoxicity evaluation conducted in this study followed the technique described by Mohd Hussain *et al.* (2022). The cytotoxic effects of the samples in the current study were assessed by procuring the supernatants and quantifying the lactate dehydrogenase (LDH) released with a cytotoxicity detection kit [Roche Applied, Burgess Hill, West Sussex, United Kingdom]. The cytotoxicity percentage (%) was calculated according to Equation (1). The control values were procured from the host cells cultured in only DMEM:

$$\text{Cytotoxicity percentage (\%)} = \frac{\text{Sample value} - \text{control value}}{\text{Total LDH released} - \text{control value}} \times 100 \quad (1)$$

The HaCaT cells treated with 2% Triton® X-100 for 1 h at 37 °C were employed to evaluate the total LDH released. Absorbance of the reduced salt, formazan (dye) was measured at 490 nm. A cytotoxicity of <10% was deemed non-toxic, while cytotoxicity between 10 and 25% was considered low toxic. Cytotoxicity between 25 and 40% was labeled intermediate, while levels over 40% were regarded as highly toxic (Lorenzo-Morales *et al.* 2010).

RESULTS

Physiological tolerance of isolated *Acanthamoeba*

The ASA.S1 segment of the 18S rRNA gene molecular analysis indicated that the *Acanthamoeba* sampled from three recreational lakes were of the T4, T5, T9, T11, T17, and T18 genotypes (Table 1). The sequences obtained in the present study were submitted to GenBank under the accession numbers OQ247939–OQ247964.

Table 1 | Sampling site, genotype, and physiological characteristics of *Acanthamoeba* isolates used in this study

Sampling site	Isolate	Genotype	Thermotolerance assay		Osmotolerance assay		Pathogenic potential
			At 37 °C	At 42 °C	0.5 M mannitol	1 M mannitol	
Biru Lake	B1	T17	+++	+++	+++	−	Low
	B2	T18	+++	+++	+++	−	Low
	B3	T17	+++	−	−	−	Low
	B4	T4	+++	+++	+++	+	High
	B5	T18	+++	++	+++	−	Low
	B6	T180	+++	+++	+++	−	Low
	B7	T17	+++	−	+++	−	Low
	B8	T4	+++	+++	+++	−	Low
	B9	T17	+++	+++	+++	−	Low
	B10	T17	+++	+++	+++	−	Low
Titiwangsa Lake	K1	T9	+++	+++	+++	++	High
	K2	T4	+++	+++	+++	++	High
	K3	T4	+++	−	+++	−	Low
	K5	T4	+++	−	+++	−	Low
	K6	T9	+++	−	+++	−	Low
	K7	T4	+++	+++	−	−	Low
	K8	T4	+++	+++	+++	++	High
	K9	T4	+++	+++	+++	++	High
Shah Alam Lake	SA1	T4	+++	+++	+++	++	High
	SA2	T5	+++	+++	−	−	Low
	SA3	T17	+++	−	+++	−	Low
	SA4	T4	−	−	−	−	None
	SA5	T11	+++	+++	+++	++	High
	SA6	T18	+++	+++	+++	−	Low
	SA7	T4	+++	−	+++	++	Low
	SA10	T4	+++	+	+++	+	High
	Reference Strain *A. castellanii* ATCC 50492	T4	+++	+++	+++	++	High

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

The physiological assay of the amoebae samples evaluated in the present study demonstrated that 84.62% (22/26) of the isolates had thermo- and osmotolerance at 37 °C and 0.5 M of mannitol. Six T4 (B4, K2, K8, K9, SA1, and SA10), one T9 (K1) and one T11 (SA5) isolate were significantly pathogenic. The samples exhibited survivability at high temperatures (42 °C) and 1 M of mannitol osmolarity. Although the reference strain (*A. castellanii* ATCC 50492) utilized in this study survived the high temperature (42 °C) and osmolarity (1 M of mannitol), fewer cells were recorded than at 37 °C and 0.5 M of mannitol.

Secretion of active serine protease by *Acanthamoeba* isolates

When compared to the 10–250 kDa protein ladder, the *Acanthamoeba* trophozoite lysates from all samples in this study displayed comparable banding patterns on gelatin zymography gels, indicating the presence of extracellular protease activity (Figure 1). The band intensities were quantified and validated using Gel Analyzer 19.1 software for accuracy. The results recorded serine proteases with molecular weights ranging from 27 to 248 kDa, while the zymography analysis documented three to eight protease bands without the protease inhibitors (Table 2).

Two T4 (K8 and SA10) and one T9 (K1) isolate recorded 33, 42, and 133 kDa of enzyme proteases. Conversely, the B4 sample documented 56 and 133 kDa of protease enzymes (Figure 1). The addition of PMSF (serine protease inhibitor) hindered trophozoite protease activities entirely. Nevertheless, incorporating NEM (cysteine protease inhibitor) and EDTA (metalloprotease inhibitor) did not result in inhibitory effects against protease enzymes secreted by the *Acanthamoeba* trophozoites lysate from all isolates. The findings validated the different secretion patterns of serine proteases in the samples.

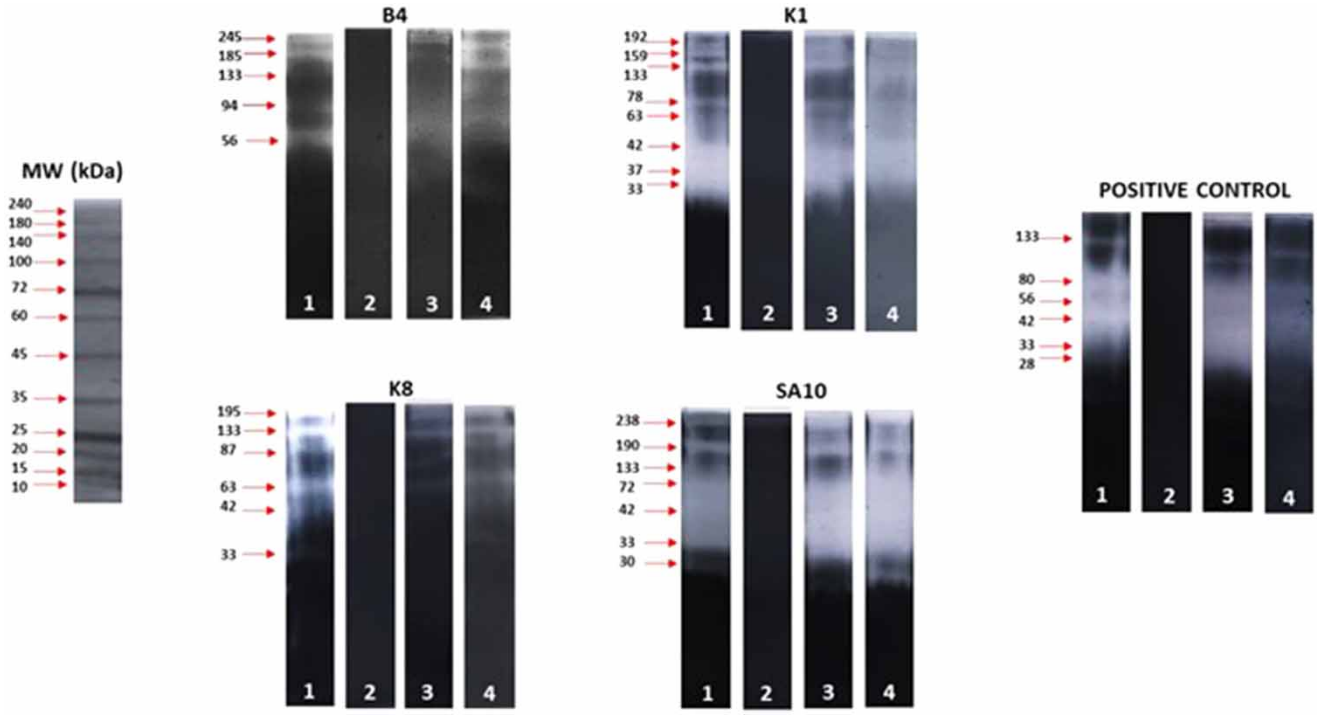


Figure 1 | Zymography analysis of *Acanthamoeba* trophozoite lysate isolates from recreational lakes without protease inhibitor (lane 1), pretreated with 1 mM PMSF (serine protease inhibitor) (lane 2), pretreated with 20 mM NEM (cysteine protease inhibitor) (lane 3), and pretreated with 10 mM EDTA (metalloprotease inhibitor) (lane 4). The molecular weight in kDa is indicated on the edge of each gel. Isolates are as follows: B4, K1, K8, and SA10.

CPEs of *Acanthamoeba* over the HaCaT cell monolayer

This study utilized the crystal violet staining technique to assess and grade the *in vitro* CPEs of the *Acanthamoeba* isolates based on the level of monolayer disruption. Figure 2 illustrates the damaging impacts of the crystal violet stain on the HaCaT cell monolayer post-incubation for 24 h. The trophozoites altered the monolayer by affixing to spaces on the plate previously occupied by HaCaT cells or between connected cells.

After 24 h, only three T4 (B4, K8, and SA10) and one T9 (K1) isolate incubated with the HaCaT cell monolayer documented cell destruction of over the 50–100% range (+++). The B4, K8, SA10, and K1 samples also recorded significant reactions toward physical stimuli. Furthermore, well plates consisting of over 50% trophozoite lysates from possibly pathogenic *Acanthamoeba* samples exhibited holes. Observably, the extracellular proteases produced by the *Acanthamoeba* isolates facilitated epithelial-cell disaggregation.

In total, 19 isolates assessed in the present study, including K2, K9, SA1, and SA5, exhibited thermo- and osmotolerance resistance. The samples demonstrated 10–50% (++) cell damage CPEs, while three isolates (K5, SA3, and SA4) recorded CPEs under 10% (+) (Table 3). Conversely, the HaCaT cell monolayer incubated with *A. castellanii* (ATCC 50492), the positive control sample documented 77.9% disruption. The monolayer without the amoebae, the negative control was the only sample without alterations.

Trophozoite lysate demonstrated *in vitro* cytopathogenicity against the HaCaT cell monolayer

The LDH results demonstrated that the potentially pathogenic *Acanthamoeba* isolates exhibited substantial *in vitro* cytopathogenicity levels in the HaCaT cell monolayer after 24 h of incubation (Table 3). Three T4 (B4, K8, and SA10) and one T9 (K1) genotype were highly toxic. The samples released over 40% LDH. Eight *Acanthamoeba* isolates, B5, B8, K2, K3, K7, K9, SA6, and SA7, recorded approximately 26–40% LDH releases, indicating intermediate cytotoxicity. Another 11 samples, B1, B2, B3, B6, B7, B9, B10, K6, SA1, SA2, and SA5 exhibited LDH releases between 11 and 25%, hence low cytotoxicity. Only three samples, K5, SA3, and SA4 released <10% LDH, which was non-toxic.

Table 2 | Protease profile (proteases number and molecular weights) of trophozoite lysate samples obtained from different isolates

No.	Isolate	Genotype	Total protease no. (MWs [kDa])	Serine protease no. (MWs [kDa])	Cysteine protease no. (MWs [kDa])	Metalloprotease no. (MWs [kDa])
1	B1	T17	5 (46, 59, 78, 159, 198)	5 (46, 59, 78, 159, 198)	–	–
2	B2	T18	5 (28, 40, 81, 168, 245)	5 (28, 40, 81, 168, 245)	–	–
3	B3	T17	6 (31, 44, 92, 128, 177, 242)	6 (31, 44, 92, 128, 177, 242)	–	–
4	B4	T4	5 (56, 94, 133, 185, 245)	5 (56, 94, 133, 185, 245)	–	–
5	B5	T18	6 (30, 45, 53, 67, 141, 249)	6 (30, 45, 53, 67, 141, 249)	–	–
6	B6	T18	5 (47, 71, 102, 140, 226)	5 (47, 71, 102, 140, 226)	–	–
7	B7	T17	6 (27, 45, 61, 83, 152, 185)	6 (27, 45, 61, 83, 152, 185)	–	–
8	B8	T4	6 (30, 57, 82, 112, 176, 234)	6 (30, 57, 82, 112, 176, 234)	–	–
9	B9	T17	6 (31, 44, 73, 117, 168, 248)	6 (31, 44, 73, 117, 168, 248)	–	–
10	B10	T17	6 (31, 45, 63, 95, 149, 238)	6 (31, 45, 63, 95, 149, 238)	–	–
11	K1	T9	8 (37, 33, 37, 42, 78, 133, 159, 192)	8 (37, 33, 37, 42, 78, 133, 159, 192)	–	–
12	K2	T4	8 (36, 44, 58, 78, 101 139, 157, 195)	8 (36, 44, 58, 78, 101 139, 157, 195)	–	–
13	K3	T4	3 (57, 101, 192)	3 (57, 101, 192)	–	–
14	K5	T4	4 (29,101, 159, 195)	4 (29,101, 159, 195)	–	–
15	K6	T9	8 (30, 35, 41, 78, 101, 127, 180, 205)	8 (30, 35, 41, 78, 101, 127, 180, 205)	–	–
16	K7	T4	5 (41, 50, 61, 101, 157)	5 (41, 50, 61, 101, 157)	–	–
17	K8	T4	6 (33, 42, 63, 87, 133, 195)	6 (33, 42, 63, 87, 133, 195)	–	–
18	K9	T4	5 (68, 78, 113, 145, 205)	5 (68, 78, 113, 145, 205)	–	–
19	SA1	T4	7 (31, 40, 54, 74, 99, 161, 242)	7 (31, 40, 54, 74, 99, 161, 242)	–	–
20	SA2	T5	4 (134, 160, 196, 235)	4 (134, 160, 196, 235)	–	–
21	SA3	T17	6 (30, 35, 44, 102, 150, 213)	6 (30, 35, 44, 102, 150, 213)	–	–
22	SA4	T4	7 (30, 39, 53, 77, 142, 173, 243)	7 (30, 39, 53, 77, 142, 173, 243)	–	–
23	SA5	T11	7 (30, 40, 53, 80, 153, 190, 233)	7 (30, 40, 53, 80, 153, 190, 233)	–	–
24	SA6	T18	7 (30, 39, 46, 69, 156, 208, 244)	7 (30, 39, 46, 69, 156, 208, 244)	–	–
25	SA7	T4	3 (30, 44, 244)	3 (30, 44, 244)	–	–

(*Continued*)

Table 2 | Continued

No.	Isolate	Genotype	Total protease no. (MWs [kDa])	Serine protease no. (MWs [kDa])	Cysteine protease no. (MWs [kDa])	Metalloprotease no. (MWs [kDa])
26	SA10	T4	7 (30, 33, 42, 72, 133, 190, 238)	7 (30, 33, 42, 72, 133, 190, 238)	–	–
27	Reference Strain *A. castellanii* ATCC 50492	T4	6 (28, 33, 42, 56, 80, 133)	6 (28, 33, 42, 56, 80, 133)	–	–

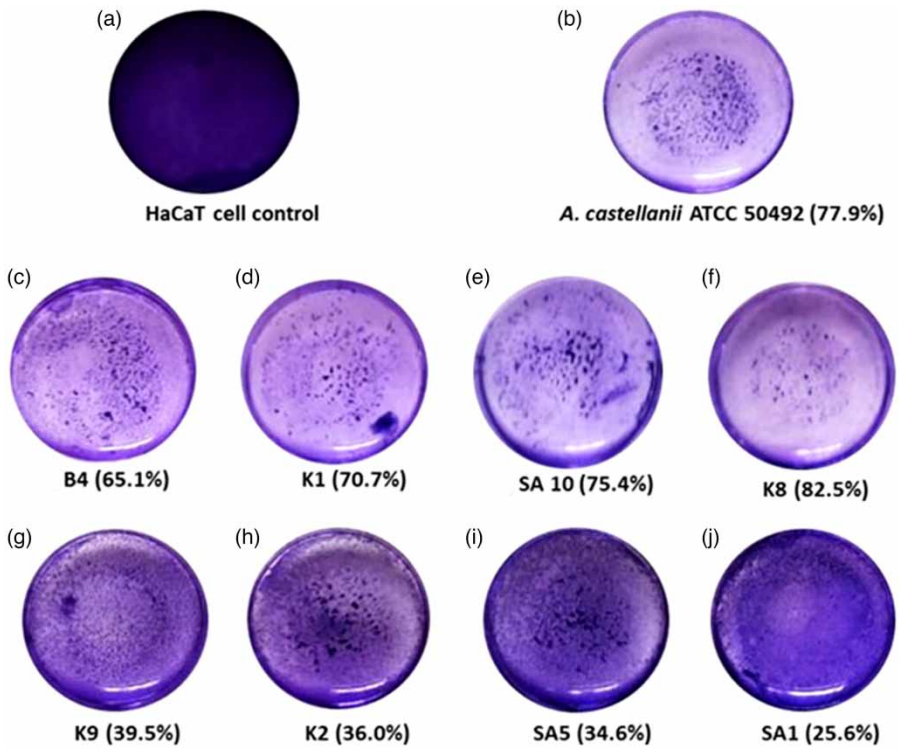


Figure 2 | Crystal violet stain demonstrating CPEs of *Acanthamoeba* isolates over the HaCaT cell monolayer. Amoebae were incubated with the HaCaT cell line in 24-well plates for 24 h at 37 °C and their CPEs were observed using the crystal violet stain. Images: (a) HaCaT cell control; (b) HaCaT cells incubated with *Acanthamoeba castellanii* (ATCC 50492) (control strain of CPE). (c)–(f) CPEs with 50–100% monolayer destruction and (g)–(i) depicts CPEs with 10–50% monolayer destruction. Images are representative of experiments performed in triplicate.

DISCUSSION

The ubiquity of the cosmopolitan *Acanthamoeba* protozoan could harm human health due to its capability to infect a host and survive in the environment. *Acanthamoeba* infections are associated with significant mortality and morbidity. Consequently, establishing the pathogen and initiating appropriate treatments immediately is critical. Nevertheless, data on the biological and cytopathogenic properties of different *Acanthamoeba* genotypes and their correlations with the virulence of each strain are limited.

The identification of *Acanthamoeba* genotypes T4, T5, T9, T11, T17, and T18 from three recreational lakes in Malaysia reflects both expected and novel ecological findings. The T4 genotype, which was frequently detected aligns with previous reports of its prevalence in diverse environments such as lakes and swimming pools and its strong association with *Acanthamoeba* keratitis infections, particularly among contact lens users (Siddiqui & Khan 2012; Rivera & Adao 2009). Genotypes T5, T9, and T11 although less commonly reported have also been isolated from aquatic environments, suggesting a diverse ecological community with varying pathogenic potentials (Booton *et al.* 2009). Notably, the presence of the rarer T17 and T18

Table 3 | Percentages of cytopathic effects and cytopathogenicity (LDH release) at 24 h after inoculation of the different *Acanthamoeba* isolates with the HaCaT cell line

			Cytopathic effect		Mean cytotoxicity level LDH	
Sampling site	**Isolate**	**Genotype**	**%**	**Grade**	**%**	**Interpretation**
Biru Lake	B1	T17	14.3 ± 1.5	++	15.6 ± 1.1	Low cytotoxicity
	B2	T18	25.7 ± 1.2	++	24.4 ± 1.7	Low cytotoxicity
	B3	T17	25.8 ± 1.1	++	21.3 ± 1.0	Low cytotoxicity
	B4	T4	65.1 ± 1.9	+++	64.7 ± 0.9	High cytotoxicity
	B5	T18	38.5 ± 1.6	++	37.6 ± 0.6	Intermediate cytotoxicity
	B6	T18	31.0 ± 1.3	++	25.1 ± 1.4	Low cytotoxicity
	B7	T17	24.5 ± 1.0	++	23.6 ± 0.6	Low cytotoxicity
	B8	T4	23.8 ± 2.8	++	29.9 ± 1.4	Intermediate cytotoxicity
	B9	T17	22.4 ± 1.0	++	21.1 ± 1.1	Low cytotoxicity
	B10	T17	10.4 ± 1.6	++	23.9 ± 1.7	Low cytotoxicity
Titiwangsa Lake	K1	T9	70.7 ± 0.5	+++	59.4 ± 1.8	High cytotoxicity
	K2	T4	36.0 ± 2.1	++	38.0 ± 1.0	Intermediate cytotoxicity
	K3	T4	36.8 ± 1.0	++	36.4 ± 0.4	Intermediate cytotoxicity
	K5	T4	8.3 ± 2.4	+	7.5 ± 0.6	Non-toxic
	K6	T9	12.0 ± 1.3	++	11.2 ± 1.3	Low cytotoxicity
	K7	T4	35.5 ± 2.1	++	38.4 ± 1.0	Intermediate cytotoxicity
	K8	T4	82.5 ± 1.9	+++	70.3 ± 0.8	High cytotoxicity
	K9	T4	39.5 ± 1.6	++	35.5 ± 1.5	Intermediate cytotoxicity
Shah Alam Lake	SA1	T4	25.6 ± 2.3	++	24.1 ± 1.2	Low cytotoxicity
	SA2	T5	13.7 ± 2.4	++	15.8 ± 1.0	Low cytotoxicity
	SA3	T17	9.0 ± 1.6	+	4.8 ± 0.8	Non-toxic
	SA4	T4	9.0 ± 0.9	+	3.9 ± 0.2	Non-toxic
	SA5	T11	34.6 ± 2.0	++	24.4 ± 1.6	Low cytotoxicity
	SA6	T18	31.6 ± 0.3	++	28.3 ± 1.4	Intermediate cytotoxicity
	SA7	T4	39.1 ± 1.3	++	36.8 ± 1.6	Intermediate cytotoxicity
	SA10	T4	75.4 ± 1.9	+++	61.4 ± 1.0	High cytotoxicity
	Reference Strain *A. castellanii* ATCC 50492	T4	77.9 ± 0.3	+++	63.5 ± 0.7	High cytotoxicity

genotypes may indicate site-specific environmental conditions or ecological niches that are underexplored, underscoring the need for further research into their distribution and pathogenic relevance (Lorenzo-Morales *et al.* 2015).

Eight T4 and singular T9 and T11 genotypes of *Acanthamoeba* isolates in this study were thermo- and osmotolerant, which are derivative virulence parameters. The findings paralleled the seven significantly thermo- and osmotolerant *Acanthamoeba* T4 isolates sampled from hot springs and beach water in Malaysia (Mohd Hussain *et al.* 2022). Predominantly, the T4 genotype obtained from *Acanthamoeba* in the environment demonstrated considerable thermo- and osmotolerance (Castro-Artavia *et al.* 2017). AK is primarily caused by genotype T4 (Satitpitakul *et al.* 2021). According to the defined sequence characteristics, T4 encompasses the T4A, T4B, T4C, T4D, T4E, T4F, and T4Neff subgroups (Fuerst & Booton 2020).

The T9 and T11 genotypes assessed in the present study were more capable of growing under harsh environments than the sample reported by Mohd Hussain *et al.* (2019). Furthermore, *Acanthamoeba* T11 isolate also demonstrated enhanced ability to grow in both extreme conditions, as reported by a number of previous studies (Todd *et al.* 2015; Milanez *et al.* 2020; Mohd Hussain *et al.* 2022). Additionally, a study carried out in Brazil discovered that *Acanthamoeba* T9 isolate was capable of flourishing in hyperosmotic and high-temperature environments, despite the fact that they were previously classified as non-pathogenic strains (Magliano *et al.* 2012). Nevertheless, it was important to highlight that, in studies conducted in Iran and India, T9 and T11 genotypes typically classified as non-pathogenic amoebae were the causal agents of AK and GAE (Hajialilo *et al.* 2016; Megha *et al.* 2018). This finding proved the pathogenic nature of the T9 genotype. Moreover, the T11 genotype which was scarcely reported worldwide was also identified in Chilean patients with AK (Jercic *et al.* 2019).

To be classified as potentially pathogenic, *Acanthamoeba* must exhibit thermo- and osmotolerant traits that reflect its behavior under stressful conditions (Todd *et al.* 2015). Their capacity to induce cellular damage *in vitro* is significantly correlated with growth at temperatures exceeding 40 °C (Walochnik *et al.* 2000), while proliferation under high mannitol concentrations indicates resistance to substantial osmotic pressure, which is critical in the corneal epithelium

(Siddiqui & Khan 2012). However, isolates from similar genotypes may display varying pathogenic potential due to adaptability to environmental conditions, influenced by the release of heat shock proteins (HSP60 and HSP70) under stress (Solgi *et al.* 2012). Importantly, the presence of these traits does not define pathogenicity. For example, Possamai *et al.* (2018) found that certain thermotolerant strains were non-pathogenic. Kahraman & Polat (2024) reported that three of four T4B and T4E genotypes isolated from keratitis cases could not thrive at 39–41 °C and 1 M of mannitol, indicating that such tolerances do not equate to virulence. Given that the human cornea maintains a temperature of 32–35 °C, the pathogenicity of amoebae cannot be accurately assessed based solely on physiological characteristics, as they may still colonize hosts despite failing to grow above 37 °C (Mohd Hussain *et al.* 2022). Therefore, comprehensive pathogenicity assessments using diverse assays, such as the CPEs are essential to elucidate the impact of each *Acanthamoeba* genotype on human infections (Mohd Hussain *et al.* 2022).

Although proteases are critical in *Acanthamoeba* biology and pathogenesis, information on the enzymes is still inadequate (Khan 2006). Only a few investigations on a limited number of isolates of varying species and genotypes have been reported on *Acanthamoeba* extracellular proteases. Accordingly, the present study evaluated the extracellular serine proteases produced by all environmental isolates.

The migration patterns obtained in this study demonstrated bands with molecular weights from 27 to 248 kDa. The results confirmed the primary proteolytic enzymes previously detected in *Acanthamoeba* (Cirelli *et al.* 2020). The findings were comparable to other *Acanthamoeba* samples, including clinical cases (Khan 2006; Castro-Artavia *et al.* 2017). Three *Acanthamoeba* T4 (B4, K8, and SA10) isolates assessed in this study had 133 kDa serine protease. The observations supported a report where 133 kDa serine protease were detected in clinical isolates (Huang *et al.* 2017).

Huang *et al.* (2017) demonstrated that 133 kDa serine protease protein resulted in cytotoxic effects on human and hamster corneal epithelial cells. A 133 kDa serine protease, MIP133 was also vital in the *Acanthamoeba* pathogenesis pathogenic cascade. The protease promotes the degradation of keratocytes and iris ciliary body, retinal pigment epithelial, corneal epithelial and corneal endothelial cells and apoptosis of macrophage-like cells (Lorenzo-Morales *et al.* 2015). The 33 kDa enzyme detected in this study is critical in corneal tissue pathogenicity and invasions. The protease or a similar enzyme was identified in *A. castellanii*, *A. healyi* and *A. lugdunensis* (de Obeso Fernandez del Valle *et al.* 2023). Kim *et al.* (2006) suggested that the 33 kDa serine protease secreted by the keratopathogenic *A. lugdunensis* is crucial to AK pathogenesis, including corneal tissue invasion, immune evasion and nutrient uptake.

Mitra *et al.* (1995) reported that the approximately 42 kDa serine protease in *Acanthamoeba* belongs to the T4 genotype. The protease could be predominantly actin, which commonly correspond to approximately 20% of the total protein in *Acanthamoeba* (Pumidonming *et al.* 2014). The 56 kDa proteolytic enzyme was also detected in this study (genotype T4). The enzyme was detected in a contact lens-wearing keratitis patient in Spain (Heredero-Bermejo *et al.* 2015).

The diverse serine proteases within a single *Acanthamoeba* genotype might be attributed to strain, virulence, culture condition or assay method variations. Dudley *et al.* (2008) proposed that serine proteases play a role in nutrition and encystment and excystment, as they are required for eventual facultative parasitism by an amoeba. Khan (2006) highlighted the direct functional role of serine proteases in *Acanthamoeba* infections, demonstrating that intrastromal injections of the organism-conditioned medium resulted in *in vivo* corneal lesions similar to those observed in AK patients with reactions inhibited by PMSF, a serine protease inhibitor. Mechanistically, serine proteases contribute to *Acanthamoeba* virulence by degrading extracellular matrix (ECM) components such as collagen and elastin, thereby facilitating tissue invasion and enhancing the amoeba's ability to penetrate host tissues. Their enzymatic activities, including collagenase and elastinolytic functions, target key structural elements in the ECM, allowing the amoeba to evade immune responses and establish infections (Siddiqui & Khan 2012). Moreover, the ability of serine proteases to degrade plasminogen further promotes ECM breakdown and inflammation, exacerbating tissue damage. This enzymatic activity is particularly critical in conditions like keratitis and GAE, especially in immunocompromised individuals (Wang *et al.* 2023).

This study utilized HaCaT keratinocyte skin cells, a frequently employed cytolytic activity model, to determine probable *in vitro* cytotoxicity and CPEs of the *Acanthamoeba* samples (Anwar *et al.* 2019, 2020). Cell viability and cytotoxicity assays assess *in vitro* modifications at the cellular and metabolic levels by detecting structural alterations, such as membrane integrity loss or physiological and biochemical reactions correlated to non-viable and viable cells (Riss *et al.* 2013). Consequently, the approaches are useful in evaluating the cytotoxic effects of the *Acanthamoeba* isolates on human cells.

In this study, HaCaT monolayer disruptions were observed with crystal violet staining. The cultures were evaluated 24 h after adding B4, K1, K8, and SA10 or the control strain, *A. castellanii* (ATCC 50492). The disaggregation of the monolayer by amoebae trophozoites affixed to the plate in the spaces previously occupied by cells or between joined cells was primarily

documented. The identical isolates and control strains also demonstrated a cytotoxicity level corresponding to the degree of cellular destruction observed in the crystal violet assay during the LDH release assessment. The study results coincided with the report by Martin-Navarro *et al.* (2010). *Acanthamoeba* Neff reportedly had 60–70% cytotoxicity, while *Acanthamoeba* CLC-16 had 55–75%.

Mohd Hussain *et al.* (2022) employed HeLa cell lines to determine cytotoxicity percentages of *Acanthamoeba* T4 (CL5, CL54, and CL149) and *Acanthamoeba* T3 (SKA5-SK35). The study reported significantly lower cytotoxicity levels, 50.9–60.6% (T4) and 50.2% (T3), than the results in the current study (59.4–70.3%). The difference could be related to the type of cell employed in the interaction.

The results in the present study suggested that the environmental samples, B4, K8, SA10 (genotype T4) and K1 (genotype T9), demonstrated an *in vitro* CPE revealing the cell monolayer disruptive abilities of the amoebae. The CPE evaluations in this study were conducted after subjecting the cultures to 12 passages, which took a maximum of 6 weeks (data unavailable). Although the procedure did not require a long monoxenic maintenance period, attenuation in CPE properties could not be discarded. Nevertheless, the detection of CPE even at low levels indicated pathogenicity, which was taken into account during analysis.

This study highlights important public health risks associated with recreational water use by identifying specific *Acanthamoeba* genotypes such as genotype T4, which is linked to infections like AK. These findings support more targeted risk assessments, helping health authorities monitor high-risk areas and populations. The currents study also provide valuable data for public awareness campaigns to educate recreational water users, particularly contact lens wearers on preventive measures. Additionally, the results can inform guidelines for water monitoring programs and safety protocols at recreational facilities. Identifying these genotypes lays the foundation for future surveillance efforts, offering insights into how environmental changes may influence the distribution and pathogenicity of *Acanthamoeba* over time.

In this study, only three recreational lakes were sampled, which may limit the generalizability of the findings regarding *Acanthamoeba* genotypic diversity. While these lakes were selected to reflect diverse human activities and environmental conditions, a broader sampling across additional lakes and geographical regions would provide a more comprehensive understanding of genotypic variation. Future research should consider expanding the scope to include lakes with different ecological conditions, human usage patterns and geographical locations. Moreover, since the data for this study were collected over a 1-year period, seasonal fluctuations and long-term trends in *Acanthamoeba* populations were not captured. This limitation may affect the interpretation of genotype prevalence and pathogenicity, as environmental conditions and human activity levels vary across seasons. Future research should consider implementing longitudinal studies over multiple years to account for seasonal variability and provide a more comprehensive understanding of *Acanthamoeba* population dynamics and potential pathogenicity.

CONCLUSION

In summary, the current study findings demonstrated virulence factors, suggesting the pathogenic potential of three *Acanthamoeba* T4 (B4, K8, and SA10) and a T9 (K1) genotype isolated from three recreational lakes in Peninsular Malaysia. Although amoebic pathogenicity could arise from intrinsic characteristics, *Acanthamoeba* infections might also indicate correlations between amoebic features, including growth temperature, proteolytic activity, and *in vitro* cytotoxicity. The present study provides novel insights and suggests a considerable variation in the response of *Acanthamoeba* members of the same genotype to physiological and biochemical pathogenicity indicators making generalization difficult. It is important to evaluate whether such differences exist among the different subtypes/species within the same genotype, especially those under genotype T4, which is implicated in the majority of *Acanthamoeba* infection. The significant LDH released (59.4%) by the environmentally isolated T9 genotype strain in the present study highlighted its infection-causing potential. The observations demonstrated that re-evaluating the role of other non-pathogenic *Acanthamoeba* genotypes in producing AK and other non-keratitis infections is necessary. The information would also provide novel and reliable diagnosis approaches and therapeutic strategies.

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AUTHOR CONTRIBUTIONS

T.S.A. and R.H.M.H. contributed to the study conception and design. T.S.A. and R.A.H. implemented the study. R.A.H. performed the experiment. R.A.H., H.H., S.A., and R.H.M.H. analyzed and interpreted the data. T.S.A., N.A.K., and R.S. revised the work critically for intellectual content and granted final approval for publishing. All authors have reviewed the manuscript and consent was given to publish.

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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