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Occurrence and molecular characterisation of *Acanthamoeba* isolated from recreational hot springs in Malaysia: evidence of pathogenic potential

Rosnani Hanim Mohd Hussain, Ahmad Razali Ishak, Mohamed Kamel Abdul Ghani, Naveed Ahmed Khan, Ruqaiyyah Siddiqui and Tengku Shahrul Anuar

ABSTRACT

This study aimed to identify the *Acanthamoeba* genotypes and their pathogenic potential in five recreational hot springs in Peninsular Malaysia. Fifty water samples were collected between April and September 2018. Physical parameters of water quality were measured *in situ* while chemical and microbiological analyses were performed in the laboratory. All samples were filtered through the nitrocellulose membrane and tested for *Acanthamoeba* using both cultivation and polymerase chain reaction (PCR) by targeting the 18S ribosomal RNA gene. The pathogenic potential of all positive isolates was identified using thermo- and osmotolerance tests. Thirty-eight (76.0%) samples were positive for *Acanthamoeba*. Water temperature ($P = 0.035$), chemical oxygen demand ($P = 0.026$), sulphate ($P = 0.002$) and *Escherichia coli* ($P < 0.001$) were found to be significantly correlated with the presence of *Acanthamoeba*. Phylogenetic analysis revealed that 24 samples belonged to genotype T4, nine (T15), two (T3) and one from each genotype T5, T11 and T17. Thermo- and osmotolerance tests showed that 6 (15.79%) of the *Acanthamoeba* strains were highly pathogenic. The existence of *Acanthamoeba* in recreational hot springs should be considered as a health threat among the public especially for high-risk people. Periodic surveillance of hot spring waters and posting warning signs by health authorities is recommended to prevent disease related to pathogenic *Acanthamoeba*.

Key words | *Acanthamoeba*, culture, genotype, hot spring, Malaysia, PCR

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INTRODUCTION

The genus *Acanthamoeba* is free-living amoebae, which is ubiquitous in natural and human-made aquatic environments, including hot spring water, tap water, lakes, ponds, freshwater sources and swimming pools (Huang & Hsu 2010). Founded on their cyst characteristics, *Acanthamoeba* can be classified into three markedly different groups (I, II and III) (Pussard & Pons 1977) and encompassing more than 25 nominal species (Booton *et al.* 2005). Furthermore, consecutive research investigating molecular and biochemical techniques have highlighted numerous examples of incongruity concerning the genotype or *Acanthamoeba* grouping (Corsaro & Venditti 2010). As a result, the genus classification and taxonomy of *Acanthamoeba* have presently been categorised into 21 diverse genotypes that are

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arranged between T1 and T21 by employing molecular methods based on 18S rRNA nucleotide sequencing (Corsaro *et al.* 2017). Potentially pathogenic T4 is the most predominant genotype isolated from corneal, skin, nasal, river, hot springs, swimming pools and beaches (Booton *et al.* 2005; Niyyati *et al.* 2016; Basher *et al.* 2018).

There are some diseases like pneumonitis, sinusitis, granulomatous amoebic encephalitis (GAE) and *Acanthamoeba* keratitis (AK) including disseminated tissue diseases which are caused by *Acanthamoeba* (Visvesvara & Schuster 2008). Consequently, there has been increasing interest by researchers in studying and identifying harmful and infectious diseases in different species of *Acanthamoeba*. In 1973, the first reported case of human infection was linked to bathing in non-sanitised water caused by AK (Illingworth & Cook 1998). Likewise, several cases have been linked to using contact lenses while swimming (Lorenzo-Morales *et al.* 2005). On the other hand, lower respiratory tract infections caused by GAE can also reach the central nervous system transmitted via the blood causing severe infection (Gianinazzi *et al.* 2009). Notably, the genus of *Acanthamoeba* can result in a consequential hazard to the well-being of humans, given it can multiply and disseminate as a carrier of infectious diseases such as *Legionella*, *Mycobacterium* and *Pseudomonas* which can withstand or tolerate extreme pH, osmolarity and temperature (Iovieno *et al.* 2010). In hotter seasons, such as in summer months, the thermotolerance of *Acanthamoeba* in water is vastly multiplied, which also includes groundwater that is naturally heated resulting in the spread of disease (Khan *et al.* 2002). Therefore, given the extent and resilience of *Acanthamoeba*, additional research and tests should be a primary concern and focus for researchers regarding its osmotolerance capability or aptitude.

Although, sadly, given the devastation caused by this disease, it has seldom been documented in Malaysia. The first AK case reported in Malaysia was in 1995 which involved a patient with a long history of wearing contact lenses (Kamel & Norazah 1995). Since the diagnosis of this first case, many AK cases have since been diagnosed indicating a dramatic increase in the aggregate of incidences over the last two decades from using contact lenses (Ghani *et al.* 2013). Nevertheless, based on the number of reported incidences of the disease caused by *Acanthamoeba*, it has also been discovered distinctly isolated from other ecological sources such as domestic tap water, seawater, swimming pools, dust from air conditioning systems and drinking water treatment plants (Ithoi *et al.* 2010; Chan *et al.* 2011; Richard *et al.* 2016). For instance, in ecological samples, T4 was frequently isolated in dry soil, dust, wet debris, wet soil and water followed by T5, T15 and T3. In the case of trauma-related AK, a particular risk factor has also been identified from wearing contact lenses where in this case, the patient had washed their eyes using water from an open tank located at a construction site (Mohamed Kamel *et al.* 2005). Indeed, there are additional factors which can pose significant risks to humans such as sand, soil, stone, etc., which can be lodged in the eyes creating frustration and causing a person to continually rub their eyes possibly incurring some damage which can invariably result in AK if left untreated (Basher *et al.* 2018).

In Malaysia, hot spring facilities, such as swimming pools, have quickly become a popular form of recreational past-time, given their ability to promote health by improving blood circulation, accelerating the healing process, soothing tired and aching muscles along with special dermatological effects (Yazdi *et al.* 2015). However, as mentioned earlier, the resilience of *Acanthamoeba* in tolerating and surviving in warm tepid temperatures in which people bathe is a significant issue and must be detected early to safeguard human health and well-being (Behets *et al.* 2007). It is important to have the capacity to rapidly detect *Acanthamoeba* in hot spring facilities where people swim and bathe, as it has become a popular pastime among people in Malaysia. To date, little serious consideration has been given to the potential for large *Acanthamoeba* infection cases in Malaysia. As a result, there is no molecular epidemiological data on the natural occurrence of *Acanthamoeba* in hot springs in this country. Hence, this study aims to provide insights into the distribution and genotypes of the potentiality pathogenic species of *Acanthamoeba* present in five hot springs in Malaysia using culture enrichment, polymerase chain reaction (PCR) amplification and DNA sequencing methods to enable taxonomic identification of the *Acanthamoeba* species. The pathogenic potential of the isolates will also be assessed employing osmotolerance and thermotolerance tests. To assist with the interpretation regarding the

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existence of *Acanthamoeba*, the parameters of physicochemical and microbiological water quality are measured.

METHODS

Collection of water samples and quality measurements

Fifty water samples, with temperatures ranging between 32 and 51 °C were initially collected from five hot springs in Malaysia: (i) Sungai Klah, Perak (3°59′ N latitude, 101°23′ E longitude), (ii) Hulu Tamu, Selangor (3°27′ N latitude, 101°41′ E longitude), (iii) Selayang, Selangor (3°15′ N latitude, 101°38′ E longitude), (iv) Bentong, Pahang (3°24′ N latitude, 101°53′ E longitude) and (v) Gadek, Malacca (2°24′ N latitude, 102°14′ E longitude) (Figure 1). The samples were collected between April and September 2018, in four regional areas of Peninsular Malaysia (Northern, East Coast, Central and Southern). The samples were taken from surface water not exceeding a depth of 10 cm. For each respective water sampling location, 1 L of water was poured into a sterile borosilicate Schott bottle, stored at 4 °C before being transported within 24 h to the Integrative Pharmacogenomics Institute, Universiti Teknologi MARA, Malaysia, for subsequent analysis. In total, 10 water samples (10 L) were collected at various sampling points from each of the hot springs.

Next, the physical parameters of the water quality were measured at each sampling location at the time of collection. The water temperature (°C), pH level, total dissolved solids (TDS) (mg/L), dissolved oxygen (DO) (mg/L) and electrical conductivity (μS/m) were measured *in situ* using a portable multiparameter (Hanna HI9828, USA). The turbidity was measured using a portable DM-TU Digimed Turbidity Meter manufactured by ITS. Additional water samples to examine chemical and microbiological indicators were also collected from each sampling location in 500 mL sterile borosilicate Schott bottles before being transported within 24 h to the laboratory in cooled containers. Testing of the chemical parameters, including the chemical oxygen demand (COD) and sulphate, was carried out in the laboratory employing the Hach method (Protocol 480, 385N and 680) and measured using a Hach spectrophotometer (HACH DR 2800™, USA). For the detection of *Escherichia*

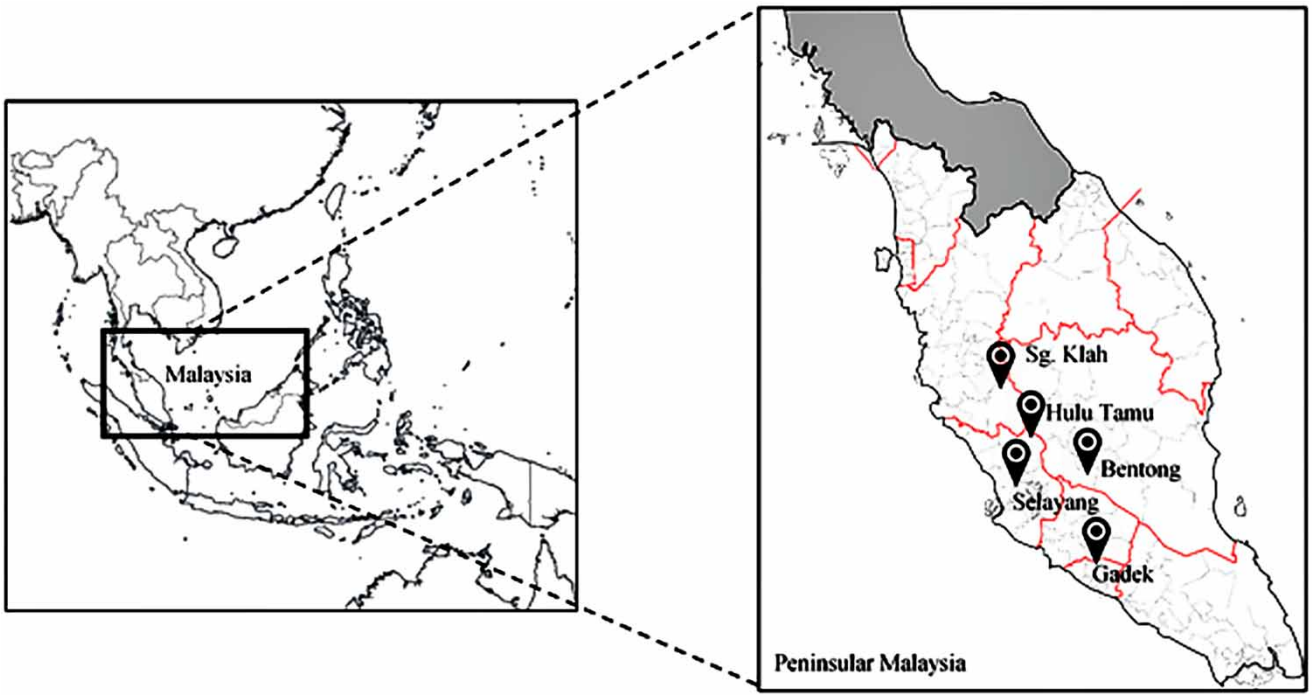

Figure 1 | A geographic map showing on the location of Peninsular Malaysia and the five sampling areas involved in the present study.

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coli (*E. coli*), Colilert® and Colilert Quanti-Tray/2000® (IDEXX, USA) were utilised based on the conventional most probable number (MPN) method (Painter *et al.* 2013) by estimating the concentration of viable microorganisms in a sample using replicate liquid broth growth in 10-fold dilutions. The principles were applied in Colilert Quanti-Tray/2000® by counting the large and small positive wells. The total positive wells were then referred to the MPN table to obtain the results.

Isolation of *Acanthamoeba* and establishment of cultures

The membrane filtration method was applied for each 1 L water sample collected utilising a sterile bottle top filter system through a cellulose 0.45 μm pore diameter nitrate filter (Gottingen, Germany), with a weak manifold vacuum system (flow rate of 1.3 mL/min). Following filtration, the membrane was then inverted and sliced into four sections and placed onto 1.5% non-nutritive agar (NNA) plates (Sigma Aldrich A7002, USA) containing Page's amoeba saline (PAS) solution lawn with UV-inactivated *E. coli*. The final pH of the PAS solution was then adjusted to 6.9. The plates were accordingly sealed with Parafilm® and incubated upside down at 30 °C, with relative humidity 85% up to 14 days (Ithoi *et al.* 2010).

During incubation, daily inspection was performed using a bright field microscope until detecting the morphological features of the *Acanthamoeba* trophozoites or cysts according to taxonomic criteria (Visvesvara & Schuster 2008). Cultures were considered as negative if they were observed to be deficient in morphological amoeba features within 3 weeks. If these amoeba features were detected, clones were produced using the migration method (Gianinazzi *et al.* 2009). In addition, to prevent fungal contamination, a block of agar (size 1 cm^2) consisting of a minimal number of amoeba was moved to the new NNA plates comprising a lawn of UV-inactivated *E. coli* at least one to three times. On this occasion, the transfer times were determined by considering the fungal growth situation. The clones were then incubated at a temperature of 30 °C for between 3 and 4 days. Additionally, no antibiotics were used during isolation nor during further culturing of the isolates.

DNA extraction, PCR amplification assay and sequence analysis

The agar plates were covered with 1 mL of PAS solution and the amoeba was carefully scraped from each plate using an L-shaped rod. The liquid containing the amoeba was then collected and transferred to an Eppendorf tube, centrifuged at 2500 rpm for 10 min. The supernatant was then removed, and the pellet was utilised for DNA extraction using a QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction manual. The DNA yield and purity were then determined using the NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) and stored at −20 °C until further use.

The PCR assay genus-specific primers set JDP1 and JDP2 used in the present study were designed for *Acanthamoeba* genotyping as previously described (Schroeder *et al.* 2001). They were established as the following sequences, 5′-GGCCCAGATCGTTTACCGTGAA-3′ and 5′-TCTCAC-AAGCTGCTAGGGGAGTCA-3′, to amplify a 450 bp fragment of the 18S rRNA stretch *Acanthamoeba*-specific amplimer ASA.S1 of *Acanthamoeba* genotypes. 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 (2×) (Qiagen, USA), 2 μL each of the oligonucleotide primers along with 20 μL of DNase-free deionised water. The cycling conditions used a temperature of 94 °C for 3 min for the initial denaturation step, followed by 35 cycles of 30 s at 94 °C for denaturation, 1 min at 57 °C for annealing, 1 min at 72 °C for extension and a final extension at 72 °C for 10 min. Positive control (*Acanthamoeba castellanii* ATCC 50492), negative DNA control (template DNA replaced with distilled water) and sample DNA were analysed in triplicates during each PCR run. The PCR products of *Acanthamoeba* were detected using gel electrophoresis on 1.5% agarose gel (Vivantis) which was carried out with 10 μL of the reaction solution. The DNA fragments were then confirmed using ethidium bromide staining (0.5 μg/mL, 10 min). A 100 bp DNA ladder (Biolabs, USA) was used as a DNA size marker.

The sequence analysis was performed using a BigDye® Terminator v.3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA). Gene trees were phylogenetically

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constructed employing neighbor-joining distance trees that produced 1,000 bootstrapped duplicates. The GenBank database was used to assign the 18S rRNA gene sequences in order for Blast searching and orientation utilising the MEGA software application, v.6.0.6 (Mega Software, Tempe, AZ, USA) (Tamura *et al.* 2013). The highest similarity percentage was then recorded to identify the species.

Thermo- and osmotolerance tests

In order to assay the pathogenicity potential of the amoebae, two tests were performed. In conducting the first test (thermotolerance), two sets of culture plates were made ready with each plate centrally positioned with a small block of NNA, previously soaked with cysts of *Acanthamoeba* isolates. Here, the first collection of plates were incubated at a temperature of 37 °C and the second collection of culture plates were incubated for 7 days at a temperature of 42 °C following cultivation. During testing, all plates were examined daily using a bright field microscope (400× magnification). The experiments were repeated in triplicate.

In order to accomplish the osmotolerance test, small agar blocks containing *Acanthamoeba* cysts were sliced and centrally positioned on a fresh 1.5% NNA containing 0.5 or 1 M of mannitol and overlaid with *E. coli*. The NNA culture plates without mannitol were used as a negative control. Then, to assess the growth, the plates were incubated for 7 days at a temperature of 30 °C. To determine any evidence of growth or nil growth during this stage, the number of cysts or trophozoites that were detected at around 20 mm from the middle of each plate were calculated and scored as 0 (−), 1–15 (+), 16–30 (++) and >30 (+++) (Landell *et al.* 2013). The experiments were executed three times and the growth after the incubation period was then assessed similarly as for the thermotolerance assay. *Acanthamoeba castellanii* (ATCC 50492) was used as a reference strain for a potentially pathogenic isolate.

Statistical analysis

To analyse the data, SPSS software (Statistical Package for Social Sciences) for Windows version 24 (SPSS, Chicago, IL, USA) was used. All water samples subjected to both cultivation and PCR were analysed descriptively in determining the prevalence rates and the distribution of *Acanthamoeba* genotypes. In comparing the occurrence of *Acanthamoeba* across the sampling sites, Fisher's exact test was employed. In addition, correlation analysis between the presence of *Acanthamoeba* and the physicochemical parameters was determined using Spearman's rho correlation coefficient (r). A probability (P) value <0.05 was considered as evidence of statistical significance.

RESULTS

Frequency of *Acanthamoeba* occurrence in hot spring recreation areas

The locations of the five hot spring recreation areas (latitude and longitude) as well as the results of *Acanthamoeba* detection are presented in Table 1. Among the 50 hot spring water samples, 38 (76%) samples were identified as positive using culture along with direct microscopy. The percentages of samples containing *Acanthamoeba* was highest in Hulu Tamu (100%), followed by Gadek (90%), Sungai Klah (70%), Bentong (70%) and Selayang (50%). The *Acanthamoeba* obtained by the culture-confirmed method are able to survive, reproduce and were usually visible within 1 week. *Acanthamoeba* revealed the presence of double-walled cysts measuring 20–25 μm (Figure 2(a)–2(d)) and trophozoites having flat shape and spine-like structures (Figure 2(e)–2(f)). It is also worth mentioning that according to the Fisher's exact test, there was no significant association between sampling sites and the occurrence of *Acanthamoeba* ($P = 0.074$).

Table 1 | Detection results of *Acanthamoeba* in five hot spring recreation areas

Sampling site	Sampling location latitude/longitude	*Acanthamoeba* percentage positivity (no. of positive/total no.) culture-confirmed method
Sungai Klah	N: 3°59′, E: 101°23′	70% (7/10)
Selayang	N: 3°15′, E: 101°38′	50% (5/10)
Hulu Tamu	N: 3°27′, E: 101°41′	100% (10/10)
Bentong	N: 3°24′, E: 101°53′	70% (7/10)
Gadek	N: 2°24′, E: 102°14′	90% (9/10)
Total		76% (38/50)

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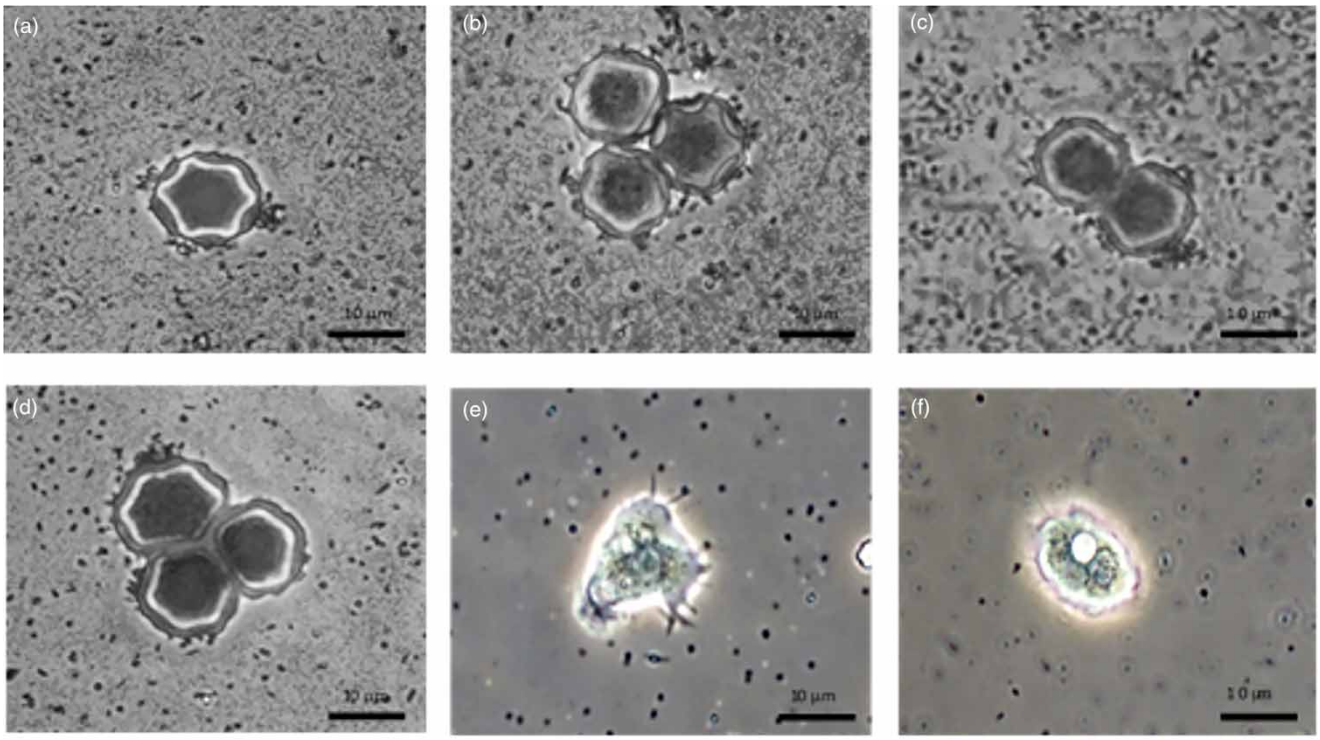

Figure 2 | Photomicrographs of representative cysts (a–d) and trophozoites (e–f) of *Acanthamoeba* isolated from hot spring recreational areas under 400× magnification.

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

Correlation between the *Acanthamoeba*-positive samples based on the culture-confirmed method and physicochemical parameters (water temperature, pH value, turbidity, DO, TDS, COD and sulphate), as well as microbiological parameter (*E. coli*) are shown in Table 2. Significant positive correlation was observed between the presence of *Acanthamoeba* with COD ($r = 0.314$; $P = 0.026$), sulphate ($r = 0.431$; $P = 0.002$) and *E. coli* ($r = 0.585$; $P < 0.001$). Nevertheless, a significant negative correlation was observed between the presence of *Acanthamoeba* and water temperature ($r = -0.299$; $P = 0.035$). No significant correlation was observed between the *Acanthamoeba*-positive samples with parameters such as DO, pH value, turbidity and TDS.

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

Water parameter	Correlation coefficient (*r*)	Significance ($P < 0.05$)
DO (mg/mL)	−0.094	0.516
Water temperature (°C)	−0.299	0.035*
pH value	0.188	0.190
Turbidity (NTU)	0.172	0.234
TDS (g/L)	0.047	0.744
COD (mg/L)	0.314	0.026*
Sulphate (mg/L)	0.431	0.002**
E. coli (MPN/100 mL)	0.585	<0.001**

*Significant at $P < 0.05$.
**Significant at $P < 0.01$.

Molecular characterisation and phylogenetic analysis of *Acanthamoeba*

Acanthamoeba-positive samples detected by culture were confirmed by PCR-based detection and then sequenced to identify the species. The *Acanthamoeba* reference strains from NCBI showed high similarity percentage (98–100%) with all 38 of the PCR products. Neighbor-joining analysis inferred the relationships between the 38 PCR products and reference strains from the NCBI GenBank, shown in Figure 3. The most frequently identified *Acanthamoeba* genotype was T4 ($n = 24$), followed by T15 ($n = 9$) and then T3

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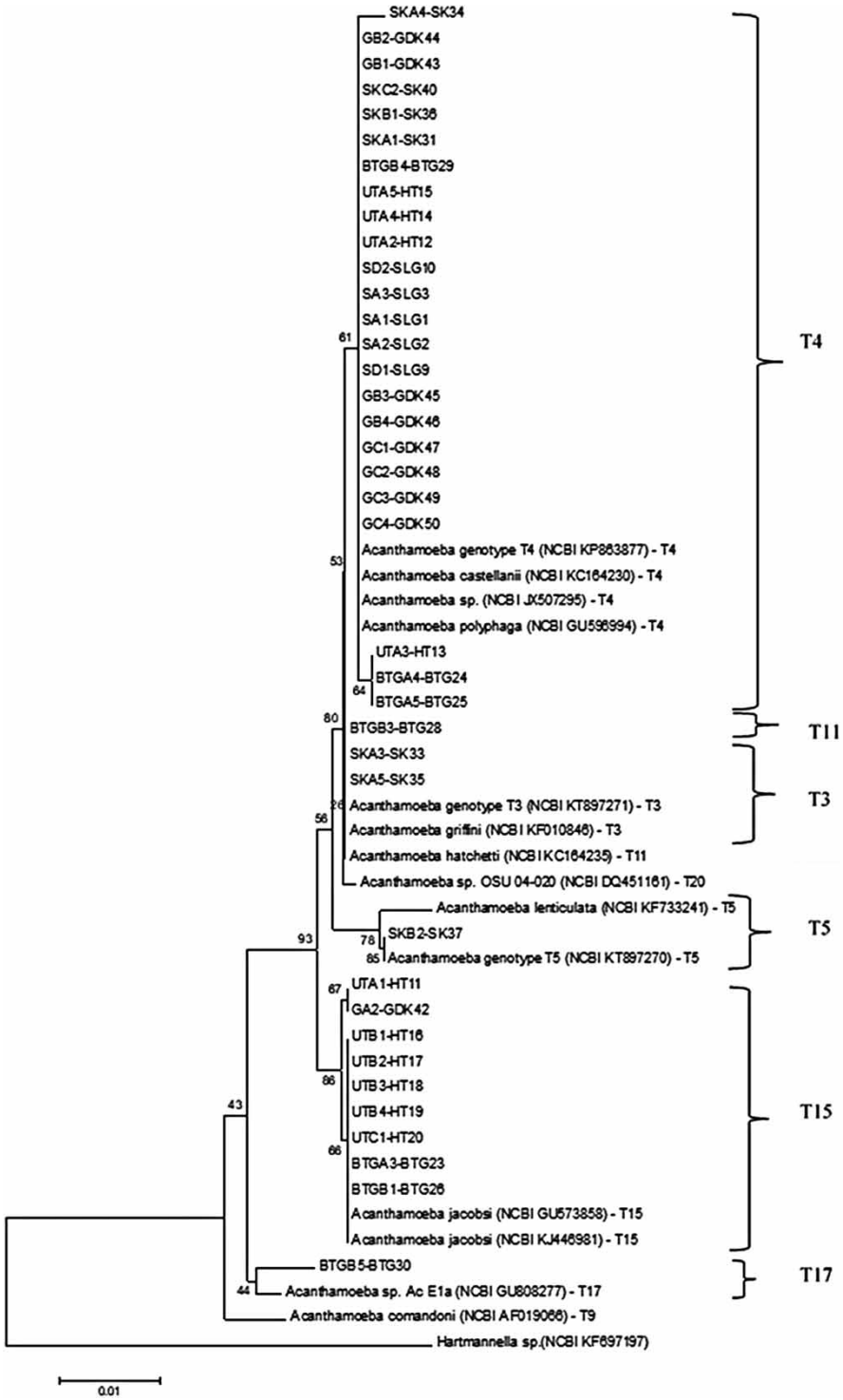

Figure 3 | Phylogenetic relationships of *Acanthamoeba* PCR products and reference strains from the NCBI GenBank inferred by neighbor-joining analysis from pairwise comparisons (450 bp fragments) of the subunit 18S rRNA nucleotide sequences in five hot spring recreation areas. *Hartmannella* sp. (NCBI KF697197) was used as an outgroup. Bar index indicates the dissimilarity (0.01) among the different sequences.

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Table 3 | Genotypic distribution of *Acanthamoeba* from five hot spring recreation areas in Malaysia

	Sampling site					
Genotype	**Sungai Klah**	**Selayang**	**Hulu Tamu**	**Bentong**	**Gadek**	**Percentage**
T3	2					5.3
T4	4	5	4	3	8	63.1
T5	1					2.6
T11				1		2.6
T15			6	2	1	23.7
T17				1		2.6

($n = 2$). Genotypes T5, T11 and T17 were detected once, respectively (Table 3). Two *Acanthamoeba* isolated from Sungai Klah detected in this study were clustered into genotype T3, the same genotype as *A. griffini* (KF010846). Twenty-four *Acanthamoeba* isolated from Sungai Klah, Selayang, Hulu Tamu, Bentong and Gadek were listed in genotype T4, similar to the species *A. castellanii* (KC164230) and *A. polyphaga* (GU596994). The other two *Acanthamoeba* that were isolated from Bentong and one from Sungai Klah were identified as genotype T11, T17 and T5, respectively. These isolates showed similar genotypes as *A. hatchetti* (KC164235), *Acanthamoeba* sp. (GU808277) and *A. lenticulata* (KT897270). Finally, nine isolates formed a cluster into genotype T15, similar to the genotype *A. jacobsi* (GU573858 and KJ446981).

All of these isolates could be a possible cause of GAE and AK. Only one isolate from Bentong was identified as *Acanthamoeba* sp. since the specific species could not be determined (Table 4). The identified genotype of *Acanthamoeba*-positive samples in the same sampling sites analysed by culture and PCR-based methods demonstrates that the *Acanthamoeba*-positive samples may include more than one *Acanthamoeba* species and genotypes. The present study possibly obtained various identified *Acanthamoeba* species and genotypes through various analytical methods. The genome sequences of the isolates were submitted to GenBank under the accession numbers MH790980–MH791016.

Pathogenic potential of *Acanthamoeba*

The response of the *Acanthamoeba* isolates from recreational hot springs towards the thermo- and osmotolerance assays is shown in Table 5. Through these tolerance tests, it was found that 6 (SD1-SLG9, UTA4-HT14, UTC1-HT20, SKA5-SK35, GB2-GDK44 and GC3-GDK49) out of the 38 isolates (15.79%) were resistant at both 37 and 42 °C temperatures including 0.5 and 1 M of mannitol. The findings also revealed that 100% of the samples tested presented thermotolerance at 37 °C. In fact, 78.94% (30/38) of the isolates managed to overcome stressful environment at 42 °C. For the osmotolerance test, only three isolates (UTA1-HT11, UTB2-HT17 and BTGA3-BTG23) were not resistant towards 0.5 M of mannitol. The reference strain (*A. castellanii* ATCC 50492) used in this study also survived

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

Genotype	**Species name**	**Sampling site**	**Associated human disease**
T3	*A. griffini*	Sungai Klah	Keratitis
T4	*A. castellanii*, *A. polyphaga*	Gadek, Selayang, Hulu Tamu, Sungai Klah and Bentong	Keratitis
T5	*A. lenticulata*	Sungai Klah	Keratitis
T11	*A. hatchetti*	Bentong	Keratitis and encephalitis
T15	*A. jacobsi*	Hulu Tamu, Bentong and Gadek	Keratitis
T17	*Acanthamoeba* sp.	Bentong	Unknown

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

			Thermotolerance assay		Osmotolerance assay	
Sample code	**Sampling site**	**Genotype**	**At 37 °C**	**At 42 °C**	**0.5 M mannitol**	**1 M mannitol**
SA1-SLG1	Selayang	T4	+++	++	++	−
SA2-SLG2	Selayang	T4	+++	++	++	−
SA3-SLG3	Selayang	T4	+++	++	+	−
SD1-SLG9	Selayang	T4	+++	++	+	++
SD2-SLG10	Selayang	T4	+++	++	++	−
UTA1-HT11	Hulu Tamu	T15	+++	+	−	−
UTA2-HT12	Hulu Tamu	T4	+++	+	++	−
UTA3-HT13	Hulu Tamu	T4	+++	+	+	−
UTA4-HT14	Hulu Tamu	T4	+++	+	++	+
UTA5-HT15	Hulu Tamu	T4	+++	−	++	−
UTB1-HT16	Hulu Tamu	T15	+++	−	++	−
UTB2-HT17	Hulu Tamu	T15	+++	−	−	−
UTB3-HT18	Hulu Tamu	T15	+++	−	++	−
UTB4-HT19	Hulu Tamu	T15	+++	−	+	−
UTC1-HT20	Hulu Tamu	T15	+++	+	+	+
BTGA3-BTG23	Bentong	T15	+++	+	−	−
BTGA4-BTG24	Bentong	T4	+++	+	++	−
BTGA5-BTG25	Bentong	T4	+++	+	+	−
BTGB1-BTG26	Bentong	T15	+++	++	+	−
BTGB3-BTG28	Bentong	T11	+++	++	+	−
BTGB4-BTG29	Bentong	T4	+++	++	+	−
BTGB5-BTG30	Bentong	T17	+++	++	++	−
SKA1-SK31	Sungai Klah	T4	+++	−	++	−
SKA3-SK33	Sungai Klah	T3	+++	++	++	−
SKA4-SK34	Sungai Klah	T4	+++	−	++	−
SKA5-SK35	Sungai Klah	T3	+++	+	+	+
SKB1-SK36	Sungai Klah	T4	+++	++	++	−
SKB2-SK37	Sungai Klah	T5	+++	+	++	−
SKC2-SK40	Sungai Klah	T4	+++	+	++	−
GA2-GDK42	Gadek	T15	+++	++	++	−
GB1-GDK43	Gadek	T4	+++	++	++	−
GB2-GDK44	Gadek	T4	+++	++	++	+
GB3-GDK45	Gadek	T4	+++	++	++	−
GB4-GDK46	Gadek	T4	+++	−	++	−
GC1-GDK47	Gadek	T4	+++	++	++	−
GC2-GDK48	Gadek	T4	+++	++	++	−
GC3-GDK49	Gadek	T4	+++	+	++	+
GC4-GDK50	Gadek	T4	+++	++	++	−
Reference strain *Acanthamoeba castellanii* ATCC 50492		T4	+++	++	+++	++

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

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at 42 °C and 1 M of mannitol but with lower number of cells than it was obtained at 37 °C and 0.5 M of mannitol.

DISCUSSION

Among the isolated free-living amoebae distributed in the environment, *Acanthamoeba* is quite common. Similarly, this protozoa is found to be present in high percentages from water environments like hot springs which are popular locations for bathing (Saburi *et al.* 2017). To the best of our knowledge, this is the first study investigating the occurrence and molecular genotyping of *Acanthamoeba* in recreational hot springs in Malaysia. *Acanthamoeba* was detected in 38 of the 50 water samples (76%) that were collected using culture and PCR-based methods. This result was in parallel with a previous study carried out locally by Latiff *et al.* (2018) also detecting a high prevalence (76%) from selected hot springs in Selangor, Malaysia, using only morphological criteria. Indeed, the higher contamination could be due to the formation of bacterial biofilm on the walls of the hot tub that attract more predatory *Acanthamoeba* that continually feed on various groups of bacteria (Khan 2006; Bagheri *et al.* 2010). For instance, several studies have been undertaken globally discovering the range of contamination in geothermally warmed waters ranging between 3.6% and 21.2%, all depending on sample size and site (Amorn *et al.* 2005; Gianinazzi *et al.* 2010; Huang & Hsu 2010; Badirzadeh *et al.* 2011).

The current study has demonstrated a significant correlation of *Acanthamoeba* with COD (mg/L), *E. coli* (MPN/100 mL), water temperature (°C) and sulphate (mg/L). High levels of COD in hot spring water will reduce the level of DO and subsequently lead to an anaerobic condition which will, in turn, cause significant harm to higher aquatic life forms, but not *E. coli*. Moreover, it will contribute to significantly high levels of bacteria in hot spring water as a source of food for *Acanthamoeba* explaining the reason why the occurrence of *Acanthamoeba* was associated with *E. coli* in the current study. Furthermore, even though *Acanthamoeba* is considered to be thermophilic protozoa, they tend to be more prevalent in hot springs having moderately low-temperature tolerance. This concurs with previous data reported at very low temperature which ultimately leads to high *Acanthamoeba* trophozoite counts, given that the optimum growth temperature is around 30 °C (Nielsen *et al.* 2014). Interestingly, *Acanthamoeba*-positive samples highlighted a significant correlation with sulphate. This study suggests that all five recreational hot springs containing sulphur may present preferable water conditions for this free-living amoeba to flourish and survive. In contrast, limited studies have indicated that the presence or absence of *Acanthamoeba* did not rely on the physicochemical parameters such as water temperature, pH value and turbidity (Tung *et al.* 2013; Richard *et al.* 2016; Ghaderifar *et al.* 2018).

In the current study, six distinct partial *Acanthamoeba* sequences belonging to the sequence T-genotype groups T3, T4, T5, T11, T15 and T17 along with six identical (*A. griffini*, *A. polyphaga*, *A. castellanii*, *A. hatchetti*, *A. lenticulata* and *A. jacobsi*) to already know *Acanthamoeba* sp. sequences from the NCBI comparisons were obtained. Only one *Acanthamoeba* sp. which was not classified to a particular species level may be unique to Malaysia. The *Acanthamoeba* strain identified as genotype T4 was reported as the predominant environmental and isolated genotype, identifying it as an important risk to the population in this country. This finding has also been supported in the literature (Niyyati *et al.* 2009; Solgi *et al.* 2012; Landell *et al.* 2013; Shokri *et al.* 2016). Moreover, *Acanthamoeba* T4 corresponding to *A. castellanii* is the primary genotype associated with AK, representing more than 90% of AK cases found in the literature (Kao *et al.* 2014). Accordingly, this suggests that recreational hot springs in Malaysia may be a significant source of acanthamoebic diseases in humans.

Nevertheless, the second genotype commonly found amid the identified *Acanthamoeba* strains was T15 with nine strains, which was clustered with *A. jacobsi*. *A. jacobsi* being sufficiently isolated from other strains of *Acanthamoeba* and is allocated to a new sequence type (T15) (Hewett *et al.* 2003). Acknowledged as a non-keratitis-initiating strain, T15 maintains its bacteriological symbionts many times more compared with other strains of *Acanthamoeba* during the cultivation or incubation process (Flint *et al.* 2003). Until recently, clinical isolates of genotype T15 were documented in Italy, representing the linkage between human amoebic keratitis and T15 (Cave *et al.* 2009; Huang & Hsu 2010).

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On the other hand, genotype T3 is less prevalent in the environment compared with T4 and T15. *A. griffini* is allocated to the T3 sequence type, which is harmful to humans and causes keratitis and infrequently GAE (Hewett *et al.* 2003). This was also evident from the collected sources of hot spring water in this study, in which *A. griffini* was found, thus suggesting that *A. griffini* is a native microorganism that thrives in these hot spring waters. Interestingly, only one isolate was detected in this study confirming the pathogenic nature of T5 in producing keratitis belonging to the T5 genotype which was found in the USA and Greece (Spanakos *et al.* 2006; Ledee *et al.* 2009). On the contrary, Huang & Hsu (2010) also reported genotype T5 as a common contaminant found in Taiwan spring recreation areas.

It is also interesting to note that six (15.8%) *Acanthamoeba* isolates were able to grow at high temperatures (42 °C) along with high osmotic stress (1 M). Likewise, 20% of the hot therapeutic springs found in north-western Iran reveal that some *Acanthamoeba* isolates which were thermotolerant are able to flourish at temperatures exceeding 40 °C (Solgi *et al.* 2012). This finding is also supported by that of Rohr *et al.* (1998), revealing that *Acanthamoeba* isolates are sensitive to temperatures exceeding 40 °C, although their ability to survive at such extreme temperatures is rare. Previous studies have also shown that *Acanthamoeba* strains that can tolerate elevated temperatures and osmolarity present the significant pathogenic potential for the well-being of both humans and animals (Visvesvara *et al.* 2007; Wannasan *et al.* 2009). Nonetheless, the *in vitro* development and growth of *Acanthamoeba* isolates at elevated temperatures or under significant osmotic stress is linked to its infectiousness, given this is partially associated with the ability of the isolate to survive and acclimatise in the host tissues of mammals (Khan & Tareen 2003). Moreover, it is important to mention that the genotypes of the mentioned strains belonging to T3, T15 and T4 are predominantly pathogenic *Acanthamoeba* which may be the result of the infectiousness of the strains undergoing environmental stress and potentially associated with aspects like the discharge of heat shock proteins (HSP70) (Solgi *et al.* 2012). However, it is proposed that further research is conducted employing *in vivo* tests which include cell culture tests or animal assays including the examination of the cytopathic consequences which, in terms of pathogenicity, are the preferred tests to be performed (Todd *et al.* 2015).

CONCLUSIONS

In summary, this study highlights the contamination of recreational hot springs in Malaysia by potentially pathogenic *Acanthamoeba* genotypes, which can result in *Acanthamoeba*-related disease. Thus, this study supports the requirement to stringently inspect recreational hot springs used for human activities (i.e. swimming) by posting warning signs at these sites to warn people about the risk associated with bathing in these waters. As a result, further study should be undertaken concerning the dissemination of this type of *Acanthamoeba* genotype in waters associated with human activities.

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