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Acanthamoeba species isolated from marine water in Malaysia exhibit distinct genotypes and variable physiological properties

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ABSTRACT

The present study identifies the *Acanthamoeba* genotypes and their pathogenic potential in five marine waters in Malaysia. Fifty water samples were collected between January and May 2019. Physical parameters of water quality were measured *in situ*, whereas chemical and microbiological analyses were conducted in the laboratory. All samples had undergone filtration using nitrocellulose membrane and were tested for *Acanthamoeba* using cultivation and polymerase chain reaction by targeting the 18S ribosomal RNA gene. The pathogenic potential of all positive isolates was identified using physiological tolerance tests. Thirty-six (72.0%) samples were positive for *Acanthamoeba*. Total coliforms ($p = 0.013$) and pH level ($p = 0.023$) displayed significant correlation with *Acanthamoeba* presence. Phylogenetic analysis showed that 27 samples belonged to genotype T4, four (T11), two (T18) and one from each genotype T5, T15 and T20. Thermo- and osmo-tolerance tests signified that three (8.3%) *Acanthamoeba* strains displayed highly pathogenic attributes. This study is the first investigation in Malaysia describing *Acanthamoeba* detection in marine water with molecular techniques and genotyping. The study outcomes revealed that the marine water in Malaysia could be an integral source of *Acanthamoeba* strains potentially pathogenic in humans. Thus, the potential risk of this water should be monitored routinely in each region.

Key words: *Acanthamoeba*, environment, free-living amoebae, genotype, Malaysia, seawater

HIGHLIGHTS

- First report on *Acanthamoeba* genotypic distribution in marine water, Malaysia.
- Highly pathogenic *Acanthamoeba* strain T4 was attributed.
- Total coliforms and pH level displayed a correlation with *Acanthamoeba* presence.

INTRODUCTION

The genus *Acanthamoeba* is a free-living opportunistic protozoan parasite frequently distributed in a variety of environments including seawater, freshwater accumulations, dam lakes, tap water, sewage, thermal waters and swimming pool water (Khan 2006; Lass *et al.* 2014). *Acanthamoeba* cyst environmental reservoirs have the potential to be transmitted to humans as well as other mammals (Edagawa *et al.* 2009; Lass *et al.* 2014). Unlike real parasites, these pathogenic amoebae may complete their lifecycle in the absence of animal or human host. Upon settling into tissues, these amoebae turn deleterious by causing severe diseases, such as granulomatous amoebic encephalitis (GAE) (affects the central nervous system and is highly fatal to the host) and amoebic keratitis (AK) (causes blindness) (Marciano-Cabral & Cabral 2003). Evidently, the high antibody titres found in human populations across the globe indicate that humans have come into contact with the pathogenic *Acanthamoeba* (Brindley *et al.* 2009). In addition to their capacity to produce damage, some *Acanthamoeba* trophozoites harbour pathogenic bacteria which are resistant to the lytic mechanisms of the amoeba such as *Legionella* sp. *Franciscella tularensis*,

Helicobacter pylori, *Mycobacterium avium* and other 'amoeba-resistant microorganisms' that exist in environmental water naturally (Siddiqui & Khan 2012). In fact, it has been postulated that these free-living amoebae could be considered biological incubators or environmental reservoirs of these bacteria, acting as 'Trojan horses' and increasing the transmission potential for humans (Janda 2010).

Identifying *Acanthamoeba* at the genus level involves detecting the characteristics of cysts and trophozoites, particularly the unique double-walled cyst shape. Initially, the *Acanthamoeba* species were categorised into three morphological groups (I, II and III) (Pussard & Pons 1977) and encompassing more than 25 nominal species (Booton *et al.* 2005). Nevertheless, the categorisation was inconsistent due to the insignificant correlation between molecular typing and binomial classification in many cases. One morphological identified species can correspond to more than one genotype and vice versa. Moreover, biochemical, growth or pathogenic characteristics and distribution have also highlighted these incoherencies in strain/species clustering (Gast *et al.* 1996; Khan & Tareen 2003). Stothard *et al.* (1998) used an 18S ribosomal RNA full gene sequence to classify *Acanthamoeba* and identified four genotypes (T1 to T4), which recorded a 5% dissimilarity value that may serve as a parameter in defining any emerging genotype. Following this classification, 22 different genotypes (T1–T22) (Tice *et al.* 2016) have been described and isolated from environmental sources worldwide, including Asian countries such as Korea, Japan, Thailand and Malaysia (Choi *et al.* 2009; Edagawa *et al.* 2009; Nuprasert *et al.* 2010; Mohd Hussain *et al.* 2019).

Although it is not always possible for the *Acanthamoeba* genotypes to be correlated with the morphologically identified species, the diversity of this genus can explain its worldwide distribution and its capability to colonise a vast range of environments. Upon assessing the *Acanthamoeba* genotypes distribution in both environmental and clinical samples, Booton *et al.* (2005) discovered that T4 was the predominant genotype in the causative agent of various diseases and in the environment. The other genotypes (T2, T3, T5, T6, T10, T12, T15 and T18) identified as pathogenic were found in clinical samples occasionally (Booton *et al.* 2005; Qvarnstrom *et al.* 2013). However, genotyping solely offers insufficient indication of an isolate pathogenicity. Commonly, *Acanthamoeba* characterisation is augmented by examining its physiological properties. Certain characteristics linked with pathogenic potential can tolerate protease activity, temperature up to 37 °C and above, cytopathic impact on cultured cells and high osmolarity (Khan *et al.* 2000). Both thermo- and osmo-tolerance tests can be deployed to distinguish non-pathogenic isolates from pathogenic ones (Lorenzo-Morales *et al.* 2005a; Costa *et al.* 2010). Upon proposing a plating assay to distinguish pathogenic from non-pathogenic isolates, Khan *et al.* (2001) found that the hallmark of pathogenic amoebae is growing at high osmolarity and temperature rates. This outcome was ascribed to the high secretion of heat shock protein in pathogenic isolates. Besides, the thermo-tolerant *Acanthamoeba* increasingly grew in geothermal-heated water and water environments in warmer climates (Mohd Hussain *et al.* 2019).

Water recreational activities, particularly in coastal areas, increase the exposure of humans to waterborne contamination. Coming into contact with coastal sediments, sewage and faeces containing pathogenic microorganisms may lead to widespread waterborne diseases that incur economic loss (Aw & Rose 2012). Therefore, rapid detection of *Acanthamoeba* in marine water is necessary, especially where coastal areas turn into a favourite holiday destination with multiple outdoor activities. Human congestion during dry weather increases the risk of *Acanthamoeba* contamination due to the easy spread of dust particles containing pathogens (Bunsuwansakul *et al.* 2019). Despite the escalating cases of AK that cause eye infection in Malaysia (Mohd Hussain *et al.* 2020), investigations that probe into the presence of *Acanthamoeba* in marine water are still scarce. As such, this present study sheds light on the genotypes and the distribution of potentially pathogenic *Acanthamoeba* across five marine waters in Malaysia. To identify *Acanthamoeba* strains, culture enrichment, polymerase chain reaction (PCR) and DNA sequencing techniques were deployed. Isolates were examined for their pathogenicity using thermo- and osmo-tolerance tests. To further interpret the detection of *Acanthamoeba*, both physicochemical and microbiological water quality parameters were determined in this study.

MATERIALS AND METHODS

Sampling sites and samples

A total of 50 surface water samples were collected from five selected marine waters in Malaysia: (i) Pantai Teluk Batik, Perak (4.1881°N, 100.6066°E), (ii) Pantai Morib, Selangor (2.7495°N, 101.4426°E), (iii) Pantai Teluk Kemang, Negeri Sembilan (2.4487°N, 101.8558°E), (iv) Pantai Tanjung Bidara, Melaka (2.2921°N, 102.0873°E) and (v) Pantai Teluk Cempedak, Pahang (3.8120°N, 103.3726°E) (Figure 1). The samples were collected between January and May 2019 across four regions

in Malaysia: Northern, East Coast, Central and Southern. All sampling sites were selected on the basis of accessibility and community presence, which evidenced anthroponotic activities. Pantai Teluk Batik in Perak for instance is located at a pleasant bay and provided a good amount of shade and a tropical atmosphere. Pantai Morib on the other hand is one of the closest beaches to Kuala Lumpur and for that reason can get busy with day-trippers during weekends and public holidays. Next, Pantai Teluk Kemang is the most popular beach destination of Port Dickson and the largest in terms of facilities and infrastructure. Pantai Tanjung Bidara has been described as a good place for relaxation, and lastly, Pantai Teluk Cempedak is attractive owing to its fantastic sea breeze.

The samples were collected from no more than 30 cm below the water surface from 10 spots along the beach. For each sampling site, a litre of sample water had been filled in a sterile borosilicate Schott bottle and kept at 4 °C prior to transportation within 48 h to the Integrative Pharmacogenomics Institute located in Universiti Teknologi MARA, Malaysia for further analysis. Permits were not required to collect water samples from public areas.

Measurement of water quality parameters

The physical parameters of water quality had been assessed *in situ* at the sampling locations during sample collection with a portable multi-parameter (Hanna HI9828, USA). The assessed parameters were electrical conductivity (μS/m), temperature (°C), dissolved oxygen (DO) (mg/L), pH level and total dissolved solids (TDS) (mg/L). Next, the turbidity level of the samples had been determined with a portable DM-TU Digimed Turbidity Meter (manufacturer: ITS). Besides, additional water samples were collected in 500 mL sterile borosilicate Schott bottles to evaluate the microbiological and chemical indicators. The samples were properly stored in an icebox with temperatures between 1 and 4 °C to preserve the majority of chemical and biological characteristics and process within less than 24 h of sample collection. The chemical parameters, namely sulphate, chemical oxygen demand (COD) and nitrogen ammonia, were assessed at the laboratory based on the Hach Method

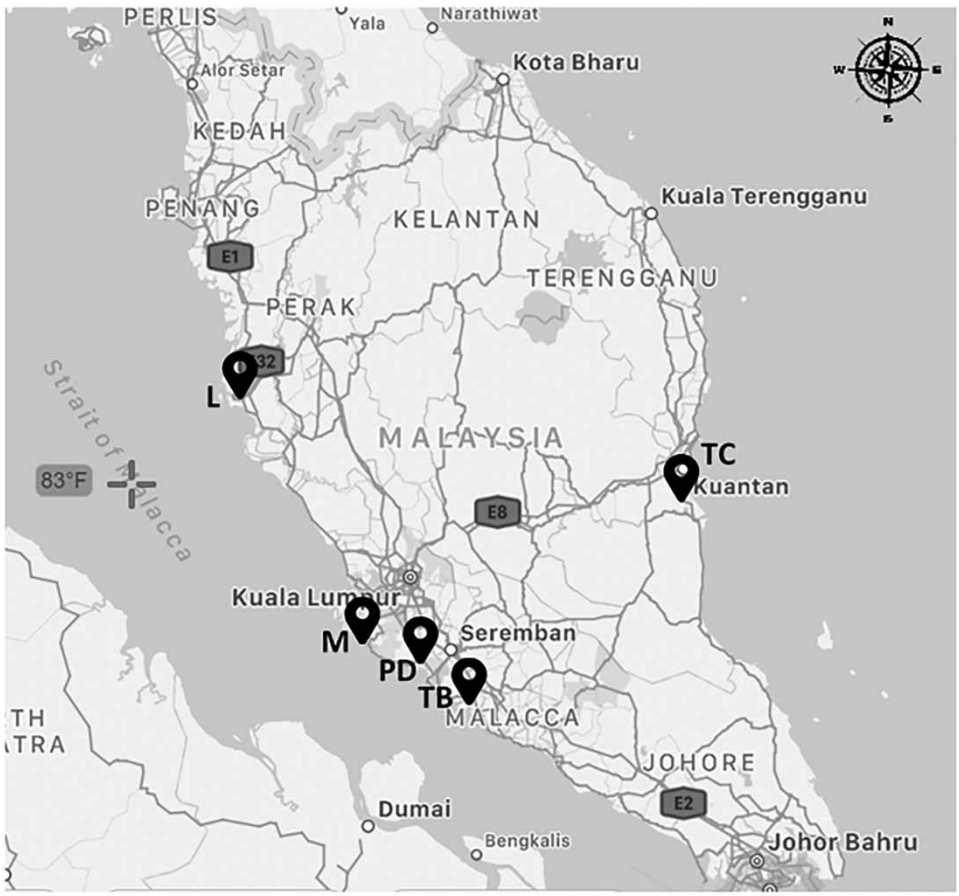

Figure 1 | Geographic map showing the location of Peninsular Malaysia and the five sampling areas involved in the present study.

(Protocol 430, 385N, and 680), and the measurements were taken with a Hach spectrophotometer (HACH DR 2800™, USA). To detect total and faecal coliform, as well as *Escherichia coli*, this study used Colilert® and Colilert Quanti-Tray/2000® (IDEXX, USA) with the most probable number (MPN) technique (Painter *et al.* 2013). This was performed by predicting the viable microorganism concentration in the samples using replicate liquid broth in 10-fold dilution. In the Colilert Quanti-Tray/2000®, large and small positive wells were counted. The MPN table served as a reference to yield the outcomes of total positive wells. The parameters obtained from this study were compared with the Malaysian Marine Water Quality Standards (MMWQS), which is a guideline on the vital standard parameters to determine the safety level of marine waters in Malaysia (DOE 2019).

Sample filtration and cultivation of *Acanthamoeba*

The membrane filtration method was applied for each 1 L water sample collected using a sterile bottle top filter system through a cellulose nitrate membrane filter (pore size: 0.45 μm) (Gottingen, Germany), with a weak manifold vacuum system (flow rate of 1.3 mL/min). Following filtration, the membrane was inverted and cut 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* K12. The final pH of the PAS solution was then adjusted to 6.9. The plates were appropriately sealed with Parafilm® and incubated upside down at 30 °C, with a relative humidity of 85% up to 14 days (Init *et al.* 2010).

Microscopic examination and culture establishment

The observation was made daily on all culture plates for 2 weeks using a light inverted microscope (Lieca DMI3000 B, Germany) before declaring negative based on the protocol prescribed by Page (1988). The *Acanthamoeba* trophozoites and cysts were detected based on the presence of spike-like pseudopodia or 'acanthapodia' size of approximately 10–25 μm, rounded or wrinkle double wall, respectively. The characteristics of cyst and trophozoite were assessed using bright-field microscope (ZEISS Primo Star, Germany) after preparing a thin smear of potentially *Acanthamoeba*-positive agar. The surface of *Acanthamoeba*-positive agar was marked with a circle using a marker pen on the bottom of the plate. Next, the lid of the plate was lifted, and the marked area on the agar surface was scraped gently with a coverslip. The gel was placed upside down and pressed evenly on a glass slide to be assessed under a bright field microscope with 1,000× magnification. Plates with positive culture were sub-cultured 10 times by placing a colony of four to six cysts onto the newly prepared NNA-*E. coli* to obtain homogeneous cell culture. Trophozoites from individual isolates were cultured in five plates to gain 5×10^3 cells. Later, they were harvested to extract total DNA with the QIAamp® DNA mini kit (Qiagen, Hilden, Germany).

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, and centrifuged at 2,500 rpm for 10 min. The supernatant was then removed, and the pellet was utilised for DNA extraction using the QIAamp® DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instruction manual. DNA yield and purity were then determined using the NanoDrop 2000c spectrophotometer (Thermo Science, USA) and stored at −20 °C until further use.

Acanthamoeba genus-specific primers, forward JDP1 (5′-GGG CCC AGA TCG TTT ACC GTG AA-3′) and reverse JDP2 (5′-TCTC ACA AGC TGC TAG GGG AGT CA-3′), were used to amplify approximately 450 bp fragment of the 18S rRNA region *Acanthamoeba*-specific amplimer ASA.S1 of *Acanthamoeba* genotypes as previously described (Schroeder *et al.* 2001). Each reaction was carried out in a final volume of 50 μL containing 25 μL of TopTaq Master Mix (2×) (Qiagen, USA), 2 μL each for the forward and reverse oligonucleotide primers, 20 μL of DNase-free deionised water and 1 μL of the DNA template (50 ng/μL). The cycling conditions were an initial denaturation of 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 s, annealing phase at 57 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. Amplification products were fractionated using 1.5% agarose electrophoresis gel (Vivantis) which was carried out with 10 μL of the reaction solution. The DNA fragments were then stained using ethidium bromide staining (0.5 μg/mL, 10 min). *Acanthamoeba castellanii* (ATCC 50492) genotype T4 and distilled water (replaced DNA template) were used as positive and negative controls, respectively.

PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. The sequencing analysis was accomplished using a BigDye® Terminator v.3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA). Direct sequencing was performed for the obtained amplicons. Multiple sequence alignment was performed using ClustalW, and the phylogenetic analyses of the dataset were performed employing neighbour-joining distance trees that produced 1,000 bootstrapped duplicates, utilising the MEGA software application, v.6.0.6 (Mega Software, Tempe, AZ, USA) (Tamura *et al.* 2013). Nucleotide sequences were identified by a BLAST search, and the highest similarity percentage was then recorded to identify the species. For phylogenetic reconstruction, sequences from all genotypes were included, and *Hartmannella vermiformis* (NCBI AF426157) was used as the outgroup.

Thermo-tolerance and osmo-tolerance tests

Two tests were conducted in this study to assay the pathogenic potential of positive samples. In the thermo-tolerance test (growth of amoebae at extreme temperatures), two culture plate sets were prepared by placing a small block of NNA (soaked with *Acanthamoeba* cysts) at the centre of the plate. Next, each plate was incubated at 37 and 42 °C for a week following cultivation. The growth of amoebas on the plates was evaluated on a daily basis with a bright field microscope (400× magnification). The experimental work was repeated in triplicate.

In the osmo-tolerance test (growth of amoebae in extreme salinity), a small agar block that contained *Acanthamoeba* cysts was sliced and centrally positioned on fresh 1.5% NNA composed of 0.5 and 1.0 M of mannitol and overlaid with *E. coli*. The NNA culture plates in the absence of mannitol served as a negative control. To examine the growth of amoebas, the plates were incubated for a week at 30 °C. Growth was evidenced based on the number of trophozoites or cysts found around 20 mm from the plate centre and rated as follows: 0 (−), 1–15 (+), 16–30 (++) and >30 (+++) (Caumo *et al.* 2009). Notably, isolates that grew at higher osmolarity and temperature were denoted as highly pathogenic strains. Low pathogens refer to the growth of amoeba detected at 37 °C and 0.5 M osmolarity (Niyyati *et al.* 2016). The experimental work was repeated thrice. Growth after the incubation period was examined similar to that used for the thermo-tolerance test. *A. castellanii* (ATCC 50492) served as the reference strain for isolates with pathogenic potential.

Statistical analyses

Statistical Package for Social Sciences software for Windows version 26 (SPSS, Chicago, IL, USA) was used to analyse the data. All water samples subjected to both cultivation and PCR were analysed descriptively in determining the detection rates and the distribution of *Acanthamoeba* genotypes. Fisher's exact test was employed to compare the frequency of *Acanthamoeba* across the sampling sites. In addition, correlation analysis between the presence of *Acanthamoeba* and the physicochemical parameters was determined using Spearman's ρ correlation coefficient (r). A probability (p) value of <0.05 was considered as evidence of statistical significance.

RESULTS

Frequency of *Acanthamoeba* occurrence in marine water

Culture results demonstrated 72% (36/50) of the water samples were positive for amoebic growth. The percentages of samples containing *Acanthamoeba* were the highest in Pantai Morib (90%), followed by Pantai Teluk Kemang (80%), Pantai Teluk Cempedak (70%) and 60% for both Pantai Teluk Batik and Pantai Tanjung Bidara. The *Acanthamoeba* obtained by the culture-confirmed method are able to survive, reproduce and were usually visible within 1 week. *Acanthamoeba* revealed a morphologic structure of cystic stages measuring 10–25 μm in diameter exhibiting star-shaped and rounded endocysts, while trophozoites demonstrated acanthopodia structure and vacuoles which compliant with morphological requirements for the *Acanthamoeba* sp. All 36 isolates from culture-positive plates were confirmed by PCR, which showed band formation between 400 and 500 bp on agarose gel (Figure 2). It is also worth mentioning that there was no significant association between sampling sites and the occurrence of *Acanthamoeba* ($p = 0.575$) based on Fisher's exact test.

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

Table 1 shows the correlation between *Acanthamoeba*-positive samples with physicochemical parameters (DO, pH value, water temperature, TDS, salinity, turbidity, COD, sulphate and nitrogen ammonia) and microbiological parameters (total coliforms, faecal coliform and *E. coli*). Findings showed that there were a significant positive correlation between *Acanthamoeba* with total coliforms ($r = 0.350$; $p = 0.013$) and a significant negative correlation between *Acanthameoba* and pH level

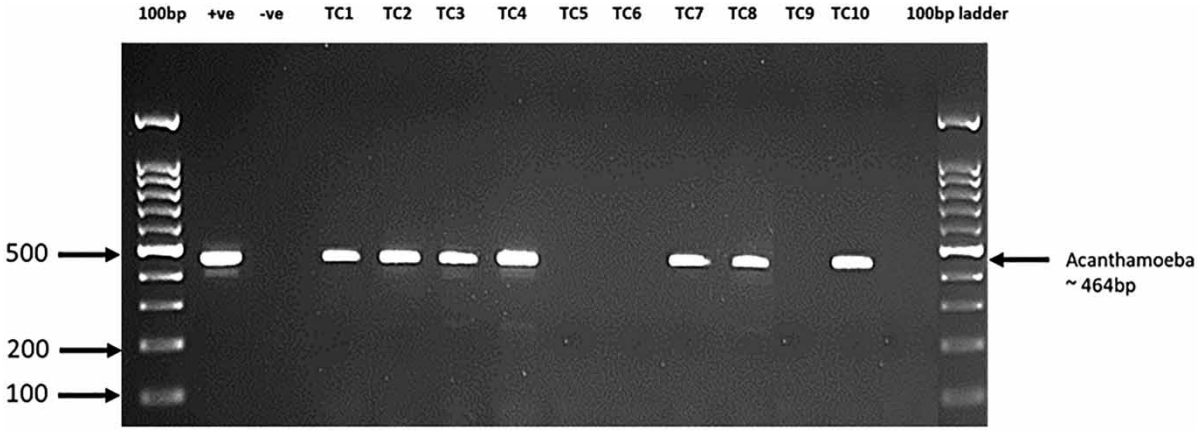

Figure 2 | PCR amplification of the isolated *Acanthamoeba* strains using JDP primers. Lane 1: 100 bp ladder was used as a molecular size marker, Lane 2: positive control *A. castellanii* (ATCC 50492), Lane 3: negative control (without DNA), Lanes 4, 5, 6, 7, 10, 11 and 13: positive cases with high load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

Table 1 | Correlation coefficient (*r*) between 12 water quality parameters and *Acanthamoeba* presence

Water parameter	Correlation coefficient (*r*)	Significance
DO (mg/mL)	0.181	0.209
pH level	−0.321	0.023*
Water temperature (°C)	−0.032	0.823
TDS (g/L)	−0.191	0.183
Salinity (psu)	−0.182	0.205
Turbidity (NTU)	0.204	0.156
COD (mg/L)	−0.147	0.309
Sulphate (mg/L)	0.023	0.873
Nitrogen ammonia (mg/L)	0.016	0.915
Total coliforms (MPN/100 mL)	0.350	0.013*
Faecal coliform (MPN/100 mL)	0.117	0.417
E. coli (MPN/100 mL)	0.096	0.508

*Correlation is significant at the 0.05 level.

($r = -0.321$; $p = 0.023$). No significant correlation was observed between *Acanthamoeba*-positive samples with other physico-chemical parameters and the presence of faecal coliform and *E. coli*.

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 a high similarity percentage (98–100%) with all 36 of the PCR products. Neighbour-joining analysis inferred the relationships between the 36 PCR products and reference strains from the NCBI GenBank, as shown in Figure 3. Twenty-seven samples from the total positive isolates were identified as genotype T4, followed by T11 ($n = 4$) and then T18 ($n = 2$). Genotypes T5, T15 and T20 were detected once, respectively (Table 2). Seven *Acanthamoeba* isolated from Pantai Morib identified in this study were clustered into genotype T4, the same genotype as *A. castellanii* (MF100900) and *A. polyphaga* (MH791016). An additional 20 *Acanthamoeba* isolated from Pantai Teluk Kemang, Pantai Teluk Batik, Pantai Tanjung Bidara and Pantai Teluk Cempedak were also recognised as genotype T4 but similar to the species *A. triangularis* (KX232518), *A. culbertsoni* (KF881887), *A. castellanii* (MF100903) and *Acanthamoeba* sp. (EU338513). Four *Acanthamoeba* genotype T11 and two genotype T18 were discovered

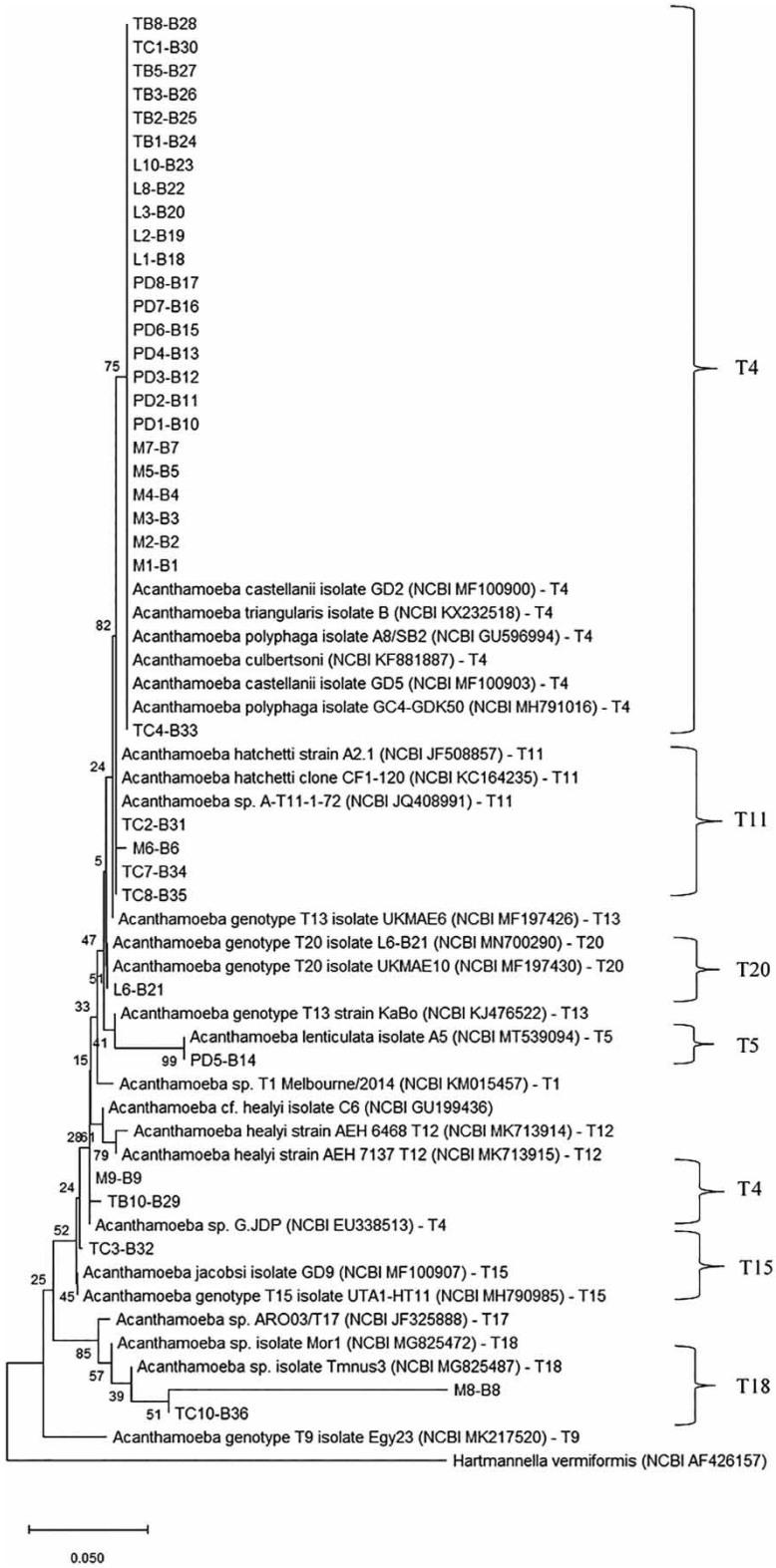

Figure 3 | Phylogenetic relationships of *Acanthamoeba* sequence inferred by neighbour-joining analysis based on the Tamura 3-parameter model produced in MEGA 6. The numbers at the branches represent the bootstrap values after 1,000 replicates. GenBank accession numbers for reference sequences are indicated at the end of the species designations. The bar index indicates the dissimilarity (0.05) among the different sequences.

Table 2 | *Acanthamoeba* genotypic distribution from five marine waters in Malaysia

	Sampling site					
Genotype	**PantaiMorib**	**Pantai Teluk Kemang**	**Pantai Teluk Batik**	**Pantai Tanjung Bidara**	**Pantai Teluk Cempedak**	**Percentage (%)**
T4	7	7	5	6	2	75
T5		1				2.8
T11	1				3	11.1
T15					1	2.8
T18	1				1	5.6
T20			1			2.8

from Pantai Teluk Cempedak and Pantai Morib. These isolates showed similar genotypes to *A. hatchetti* (JF508857) and *Acanthamoeba* sp. (MG825487). Finally, the other three isolates formed a cluster into genotypes T5 (Pantai Teluk Kemang), T15 (Pantai Teluk Cempedak) and T20 (Pantai Teluk Batik). These isolates showed similar genotypes to *A. lenticulata* (MT539094), *A. jacobsi* (MF100907) and *Acanthamoeba* sp. (MF197430).

All of these isolates could be a possible cause of *Acanthamoeba* keratitis and GAE. One isolate each from Pantai Morib, Pantai Teluk Cempedak and Pantai Teluk Batik was identified as *Acanthamoeba* sp. since the specific species could not be determined (Table 3). 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 genotype. The present study possibly obtained various identified *Acanthamoeba* species and genotypes through various analytical methods. The DNA sequences of *Acanthamoeba* isolates generated from this study were deposited in the GenBank database and are available under accession numbers from MN700270 to MN700305.

Pathogenic potential of *Acanthamoeba* isolates

The response of the *Acanthamoeba* isolates from marine water towards the thermo- and osmo-tolerance tests is shown in Table 4. Through these tolerance tests, it was found that three (M5-B5, L1-B18 and TB5-B27) out of the 36 positive isolates (8.3%) showed fast growth at both 37 and 42 °C temperatures including 0.5 and 1 M of mannitol. The aforementioned isolates were considered as highly pathogenic amoeba. The findings also revealed that 97.2% (35/36) of the samples tested presented thermo-tolerance at 37 °C. In fact, only three of the isolates managed to overcome stressful environment at 42 °C. For the osmo-tolerance test, only five isolates (M9-B9, PD1-B10, PD2-B11, L6-B21 and TC1-B30) and three isolates (M9-B9, PD2-

Table 3 | List of various *Acanthamoeba* genotypes and species isolated in this study and associated diseases

Genotype	**Species name**	**Sampling site**	**Associated human disease**
T4	*A. castellanii*	Pantai Morib, Pantai Teluk Kemang, Pantai Teluk Batik, Pantai Tanjung Bidara and Pantai Teluk Cempedak	Keratitis
	A. polyphaga	Pantai Morib, Pantai Teluk Batik and Pantai Tanjung Bidara	
	A. triangularis	Pantai Teluk Kemang	
	A. culbertsoni	Pantai Teluk Batik	
	Acanthamoeba sp.	Pantai Morib, Pantai Teluk Batik, Pantai Tanjung Bidara and Pantai Teluk Cempedak	
T5	*A. lenticulata*	Pantai Teluk Kemang	Keratitis
T11	*A. hatchetti*	Pantai Teluk Cempedak, Pantai Morib	Keratitis and GAE
T15	*A. jacobsi*	Pantai Teluk Cempedak	Keratitis
T18	*Acanthamoeba* sp.	Pantai Teluk Cempedak, Pantai Morib	GAE
T20	*Acanthamoeba* sp.	Pantai Teluk Batik	Unknown

Table 4 | *In vitro* growth of *Acanthamoeba* isolated from marine water at different temperatures and osmolarities

Sample code	Sampling site	Genotype	Score for growth[a]			
			37 °C	42 °C	0.5 M mannitol	1 M mannitol
M1-B1	Pantai Morib	T4	++	−	+	+
M2-B2	Pantai Morib	T4	++	−	+	++
M3-B3	Pantai Morib	T4	++	−	++	++
M4-B4	Pantai Morib	T4	+	−	+	+
M5-B5	Pantai Morib	T4	+++	+	++	+++
M6-B6	Pantai Morib	T11	++	−	+	++
M7-B7	Pantai Morib	T4	++	−	++	++
M8-B8	Pantai Morib	T18	++	−	+	+
M9-B9	Pantai Morib	T4	+	−	−	−
PD1-B10	Pantai Teluk Kemang	T4	+	−	−	+
PD2-B11	Pantai Teluk Kemang	T4	+	−	−	−
PD3-B12	Pantai Teluk Kemang	T4	+	−	+	+
PD4-B13	Pantai Teluk Kemang	T4	+	−	++	++
PD5-B14	Pantai Teluk Kemang	T5	+	−	+	+
PD6-B15	Pantai Teluk Kemang	T4	++	−	+	+
PD7-B16	Pantai Teluk Kemang	T4	+	−	+	+
PD8-B17	Pantai Teluk Kemang	T4	++	−	+	+
L1-B18	Pantai Teluk Batik	T4	+++	+	+	++
L2-B19	Pantai Teluk Batik	T4	+	−	++	++
L3-B20	Pantai Teluk Batik	T4	+	−	++	++
L6-B21	Pantai Teluk Batik	T20	−	−	−	−
L8-B22	Pantai Teluk Batik	T4	+	−	+	+
L10-B23	Pantai Teluk Batik	T4	+	−	+	++
TB1-B24	Pantai Tanjung Bidara	T4	++	−	++	++
TB2-B25	Pantai Tanjung Bidara	T4	++	−	+	+
TB3-B26	Pantai Tanjung Bidara	T4	++	−	+	+
TB5-B27	Pantai Tanjung Bidara	T4	++	+	++	++
TB8-B28	Pantai Tanjung Bidara	T4	+	−	+	+
TB10-B29	Pantai Tanjung Bidara	T4	++	−	+	+
TC1-B30	Pantai Teluk Cempedak	T4	++	−	−	+
TC2-B31	Pantai Teluk Cempedak	T11	+	−	+	+
TC3-B32	Pantai Teluk Cempedak	T15	+	−	+	+
TC4-B33	Pantai Teluk Cempedak	T4	+	−	+	+
TC7-B34	Pantai Teluk Cempedak	T11	+	−	+	+
TC8-B35	Pantai Teluk Cempedak	T11	+	−	+	+
TC10-B36	Pantai Teluk Cempedak	T18	+	−	+	+
Reference strain *A. castellanii* (ATCC 50492)		T4	+++	++	+++	++

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

B11 and L6-B21) were susceptible towards 0.5 and 1 M of mannitol, respectively. The reference strain (*A. castellanii* ATCC 50492) used in this study also survived at 42 °C and 1 M of mannitol but with a lower number of cells than were obtained at 37 °C and 0.5 M of mannitol.

DISCUSSION

The presence of *Acanthamoeba* species in both natural and artificial environments reflects their high adaptability to multiple conditions (Siddiqui & Khan 2012). Hence, the unavoidable exposure to *Acanthamoeba* can cause detrimental effects on one's health. The increasing recreational activities that involve marine water are a concern in light of health deterioration amongst locals and tourists (Fewtrell & Kay 2015). This study is the first investigation in Malaysia to describe the molecular genotyping and prevalence of *Acanthamoeba* in marine water samples. The *Acanthamoeba* was identified in 36 water samples (72%) out of 50 based on PCR and culture techniques. This shows that marine water in the studied sites was indeed a vital habitat of *Acanthamoeba* species, which is alarming due to the human activities noted at the Malaysian beaches all year round. This finding is in line with that claimed by Todd *et al.* (2015), who also identified a high prevalence (64%) of *Acanthamoeba* in marine water. A recent study by Mahmoudi & Karanis (2020) reported that *Acanthamoeba* spp. were identified in 50% (15/30) of the seawater samples from the Caspian Sea, Iran by culture in NNA media. At a global scale, studies had reported that marine water contamination ranged from 40–50% (Lorenzo-Morales *et al.* 2005a, 2005b). The World Health Organization (2006) detected *Acanthamoeba* in marine water contaminated with waste effluents and sewage. For instance, Sawyer *et al.* (1977) isolated *A. culbertsoni* and *A. hatchetti* from the sewage-spoil dump and the Baltimore Harbour in Maryland, respectively. Pollution also occurs due to direct wastewater disposal into seawater and beach sand with partial treatment or without treatment (Hilles *et al.* 2014).

This present study found that *Acanthamoeba* was significantly correlated with total coliforms and pH level. The pH recorded in this study ranged from 6.5–9.0, which adhered to the recommended standard for marine water pH (DOE 2019). The negative correlation indicated that the lower the pH, the higher the chances for *Acanthamoeba* to be present in marine water. Similarly, Aqeel *et al.* (2013) reported that a neutral pH of 7.0 and a higher osmolarity level reflected an optimum condition for the encystation of *Acanthamoeba*. The alkalinity of marine water is caused by dissolved basic minerals and changes that occur in environmental conditions, including pH that promotes the conversion of *Acanthamoeba* into a dormant cyst that can remain viable for years (Guimaraes *et al.* 2016). This study, nonetheless, deviates from the findings reported for Japan and Taiwan, which documented an insignificant correlation between pH and *Acanthamoeba* presence in water (Kawaguchi *et al.* 2009; Kao *et al.* 2011). Studies concerning microbiological loads in Malaysian marine water are scarce. One component that is vastly applied to measure water source cleanliness refers to total coliforms. Its presence reported in this study had been low (793 MPN/100 mL) when compared to the guidelines for recreational marine water deployed by various US governmental agencies at a geometric mean of 1,000 MPN/100 mL (U.S. EPA 1986). This is in parallel with previous data that stated a higher mean value (12–2.85×10^4 CFU/100 mL) of total coliforms induced with the presence of *Acanthamoeba* (Kao *et al.* 2011). Total coliforms generated by surface water biofilms or by the natural inhabitants could lead to *Acanthamoeba* predation. This denotes the significant correlation between *Acanthamoeba* presence and total coliforms (Kao *et al.* 2011). According to the MMWQS, the acceptable faecal coliform count is 70 MPN/100 mL (DOE 2019). Based on this standard, none of the investigated marine water appears safe for body contact (129 MPN/100 mL). Despite being statistically insignificant with the presence of *Acanthamoeba*, faecal coliform reading had exceeded the range that signifies increased risk of the public suffering from gastroenteritis and dermatitis infection (Graczyk *et al.* 2010).

In the present study, six distinct partial *Acanthamoeba* sequences that belonged to T4, T5, T11, T15, T18 and T20, along with seven identical (*A. castellanii*, *A. polyphaga*, *A. triangularis*, *A. culbertsoni*, *A. lenticulata*, *A. hatchetti* and *A. jacobsi*) to already know *Acanthamoeba* sp. sequences were detected by comparing NCBI. Meanwhile, three *Acanthamoeba* sp. unclassified to any particular species level appeared unique to the Malaysian context. The predominance of *Acanthamoeba* strain with genotype T4 increases the health risk to humans. Genotype T4 reported in this study comprised some strains and species, which, respectively, were 98 and 100% homologous with *A. castellanii*, *A. polyphaga*, *A. triangularis* and *A. culbersoni*. Similarly, other studies demonstrated that the T4 genotype is commonly detected in both wet and dry environments across the globe (Niyyati *et al.* 2009; Rahdar *et al.* 2012). This genotype is also commonly reported in clinical cases, particularly among patients diagnosed with GAE and AK (Booton *et al.* 2002; Yera *et al.* 2007). According to Booton *et al.* (2005), 72% of AK cases were due to *A. castellanii* (T4) found in 53% of samples. This highlights that marine water in Malaysia is a potential source of *Acanthamoeba* ailments in humans.

Meanwhile, T11 (with four strains) was the second genotype detected in the *Acanthamoeba* strains clustered with *A. hatchetti*. Likewise, Milanez *et al.* (2020) reported the presence of *A. hatchetti* belonging to genotype T11 in samples taken from two major water reservoirs in the Philippines. *A. hatchetti* was first isolated from brackish and oceanic sediments

of the Atlantic Ocean by Sawyer *et al.* (1977). Since the predominance of AK is still rare when linked with T11, more epidemiological analysis is sought to verify the correlation between T11 and clinical cases. A past study had isolated *Acanthamoeba* T11 from Iranian clinical cases (Niyyati *et al.* 2009). Meanwhile, a genotype rarely found, T18, was found in samples (5.6%) collected from Pantai Morib and Pantai Teluk Cempedak. Interestingly, this happened to be the initial detection of T18 in Malaysian marine water. This particular genotype has been isolated so far from brain and lungs (Matsui *et al.* 2018) and also from sewage and rivers (Possamai *et al.* 2018).

Genotype T5 (isolated from Pantai Teluk Kemang) displayed 100% homology with *A. lenticulata* infrequently found in the aquatic environment, which contradicted a local finding reported by Basher *et al.* (2018). The preferred habitat of genotype T5 is a typically wet or moist condition, such as central air conditioning (Chan *et al.* 2011) and nasal mucosa cavity (Cabello-Vilchez *et al.* 2013). This genotype could cause AK (Ledee *et al.* 2009) in immunosuppressed individuals and disseminated infection as such that was reported in a case involving a heart transplant patient (Barete *et al.* 2007). Thus, the presence of genotype T5 in aquatic environments may be a crucial reason for *Acanthamoeba* infection to those keen in water activities, particularly amongst immunosuppressed individuals. As for this present study, only one (2.8%) isolate was detected for the sample from Pantai Teluk Cempedak with *A. jacobsi* cluster. This is because genotype T15 is rarely present in non-water domestic environments (Corsaro *et al.* 2017). The *A. jacobsi* is well segregated from *Acanthamoeba* strains, thus enabling the allocation of T15 – a new genotype sequence (Hewett *et al.* 2003). The clinical isolate of T15 detected in Itali was the initial link of T15 with AK diagnosed in humans (Di Cave *et al.* 2009).

The variability of *Acanthamoeba* and its genotypes has been highlighted in their response to pathogenicity assays, thus making *in vitro* assessment difficult to correlate with actual human-pathogenic capacity (Tawfeek *et al.* 2016). Turning to this study, only three (8.3%) *Acanthamoeba* genotype T4 had grown at high temperature (42 °C) and osmotic stress (1 M), signifying the indirect factors linked with pathogenic potential (Khan 2001). On the contrary, Ettinger *et al.* (2003) found that the pathogenic potential of *Acanthamoeba* was 40.4% of seawater strains based on physiological tolerance assays in Virginia, USA. The presence of both non-pathogenic and pathogenic species in *Acanthamoeba* genus was studied by Howe *et al.* (1997), and it called for more pathogenicity assessments on *Acanthamoeba* species by employing a vast range of assays, so as to verify the impact of each genotype on human infections. Evidently, this study had probed into the interspecies variation to assess the pathogenic potential of *Acanthamoeba* genotypes via osmo- and thermo-tolerance tests. Essentially, this present study has emphasised the inability of some pathogenic genotypes (T5, T11, T15 and T18) to thrive at higher temperature and osmolarity.

CONCLUSION

In summary, the study showed that distinct *Acanthamoeba* genotypes with varying levels of pathogenicity were present in the investigated marine waters in Malaysia, which can result in *Acanthamoeba*-related disease. Since the attractiveness of recreational places for people is increasing, the public should be alerted about the risks of potential waterborne pathogens and the marine water quality must be periodically monitored. Hence, more environmental samples must be examined by including various water sources and locations. Ultimately, more studies should assess the diversity, the prevalence, and the pathogenic potential of *Acanthamoeba* genotypes.

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AUTHORS' CONTRIBUTIONS

The concept study was conducted and designed by T.S.A., M.K.A.G., N.A.K. and R.S. R.H.M.H. performed the samples collection and laboratory experiments. T.S.A. and R.H.M.H. were responsible for the analysis and interpretation of the data. T.S.A. and R.H.M.H. wrote and drafted the manuscript. N.A.K., R.S. and M.K.A.G. reviewed and edited the manuscript, and then gave the final approval for the version to be published. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

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

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