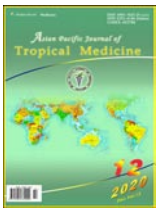

Original Article

Asian Pacific Journal of Tropical Medicine

journal homepage: www.apjtm.org

doi: 10.4103/1995-7645.296722

Impact Factor: 1.94

Morphological and molecular characterization of *Acanthamoeba* isolated from contact lens paraphernalia in Malaysia: Highlighting the pathogenic potential of T4 genotype

Rosnani Hanim Mohd Hussain[1], Nur Syahirah Mohamad Isa[1], Khairul Ameera Kamaruddin[1], Mohamed Kamel Abdul Ghani[2], Naveed Ahmed Khan[3], Ruqaiyyah Siddiqui[3], Tengku Shahrul Anuar[1,4]✉

[1]*Centre of Medical Laboratory Technology, Faculty of Health Sciences, Universiti Teknologi MARA, Puncak Alam Campus, Selangor, Malaysia*

[2]*Programme of Biomedical Science, School of Diagnostic and Applied Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, Kuala Lumpur, Malaysia*

[3]*Department of Biology, Chemistry and Environmental Sciences, College of Arts and Sciences, American University of Sharjah, University City, Sharjah, United Arab Emirates*

[4]*Integrative Pharmacogenomics Institute, Universiti Teknologi MARA, Puncak Alam Campus, Selangor, Malaysia*

ABSTRACT

Objective: To determine the morphological and molecular characterization of *Acanthamoeba* isolates from contact lens paraphernalia in Malaysia and to investigate their pathogenic potential based on the physiological tolerance.

Methods: One hundred and eighty contact lens wearers donated their contact lens, lens storage cases and lens solutions between 2018 and 2019. The samples were inoculated onto 1.5% non-nutrient agar plates for 14 d. Polymerase chain reaction (PCR) was performed and the amplified PCR products were sequenced and compared with the published sequences in GenBank. The pathogenic potential of positive isolates was further tested using temperature-tolerance and osmo-tolerance assays. *Acanthamoeba* species were categorized into three distinct morphological groups established by Pussard and Pons.

Results: *Acanthamoeba* was successfully isolated from 14 (7.8%) culture-positive samples in which 11 belong to morphological group Ⅱ and 3 belong to morphological group Ⅲ, respectively. The sequencing of *18S* ribosomal RNA gene led to the identification of the T4 genotype in all the isolated strains. *In vitro* assays revealed that 9 (64.3%) *Acanthamoeba* isolates were able to grow at 42 ℃ and 1 M mannitol and were thus considered to be highly pathogenic.

Conclusions: To the best of our knowledge, this is the first report identifying the *Acanthamoeba* genotype and their pathogenic potential among contact lens wearers in Malaysia. The potentially pathogenic T4 genotype isolated in this study is the most predominant genotype responsible for human ocular infection worldwide. Hence, increasing attention should be aimed at the prevention of contamination by *Acanthamoeba* and the disinfection of contact lens paraphernalia.

KEYWORDS: *Acanthamoeba*; Contact lens; Culture; Pathogenic; PCR; T4 genotype; Malaysia

1. Introduction

The genus *Acanthamoeba* has been isolated from diverse of environments such as water, soil, dust, air, contact lenses and their lens storage cases as well as identified as contaminants in

✉To whom correspondence may be addressed. E-mail: tengku9235@uitm.edu.my

How to cite this article: Mohd Hussain RH, Mohamad Isa NS, Kamaruddin KA, Abdul Ghani MK, Khan NA, Siddiqui R, et al. Morphological and molecular characterization of *Acanthamoeba* isolated from contact lens paraphernalia in Malaysia: Highlighting the pathogenic potential of T4 genotype. Asian Pac J Trop Med 2020; 13(12): 542-549.

Article history: Received 20 January 2020 Revision 10 April 2020
Accepted 24 April 2020 Available online 19 October 2020

yeast, bacterial and mammalian cell cultures[1]. During the last few decades, this ubiquitous amphizoic amoebae has become increasingly recognized as a causative agent of *Acanthamoeba* keratitis, an acute vision-threatening corneal infection that is often associated with contact lens misuses[2] and granulomatous amoebic encephalitis, a serious and usually fatal brain infection that occurs mainly in immunocompromised patients[3]. To date, more than 21 *Acanthamoeba* species have been described and assigned to one of the three distinct morphological groups (Ⅰ, Ⅱ and Ⅲ) established by Pussard and Pons[4] according to the size and shape of their cysts. Several other criteria such as tolerance to temperatures and high osmolarity or cytopathic effects on cultured cells play a key role in differentiating the species as well as ascertaining their pathogenic potential[5].

The classification of *Acanthamoeba* based on their morphological characteristics has been rather ambiguous *via* microscope, thereby leading to several revisions. Hence, molecular studies focusing on the small-subunit *18S* ribosomal RNA (*18S* rRNA) gene sequences are essential not only for taxonomic purposes but also for clinical and epidemiological studies relating to this species. So far, 21 genotypes (T1-T21) for *Acanthamoeba* have been described using this classification[6]. Based on the distribution of these genotypes, studies have identified the T4 as the most common genotype present in the environment or being the causative agent of various diseases[7,8]. For example, more than 90% of *Acanthamoeba* keratitis cases were found to be associated with this genotype. Similarly, T4 has also been linked with the non-keratitis infection such as granulomatous amoebic encephalitis and cutaneous infections, although the cases are quite limited[9]. At present, the reason for T4 isolates are most abundant in human infections remains unclear, although it is likely to be due to their stronger virulence properties that enhance transmissibility and decreased susceptibility to chemotherapeutic agents.

Since the first report of *Acanthamoeba* keratitis in 1974, there has been a dramatic increase in the number of *Acanthamoeba* keratitis cases over the last 20 years, with over 3 000 cases occurring in the United States alone[10]. Some of the identified factors that pose a higher risk for eye infections appear to be associated with poor hygiene practices of contact lens wearers which include failure to comply with the recommended disinfection and cleaning procedures, rinsing contact lens with homemade saline solutions or tap water, wearing lenses in the shower and the overuse of disposable contact lens[11]. The relationship between contact lens wearers and the diagnosis of *Acanthamoeba* keratitis has been demonstrated in several studies, thereby shifting the focus towards contact lens and their storage cases. Variable contamination rates have been identified in contact lens or lens storage cases according to the different geographic regions and experimental methods used. For example, studies performed using cell culture methods showed contamination rates of 4.2%-15.1% in certain regions of South Korea[12], 9% in the south of Brazil[13], 10% in Iran[14] and 65.9% in the Canary Islands, Spain[15].

In Malaysia, *Acanthamoeba* keratitis is considered a rare disease and the first case was reported in 1995 involving a woman who had a long history of using contact lens[16]. Subsequently, several cases associated with the disease were reported among those wearing contact lens. A local study conducted among contact lens wearers in Kuala Lumpur[17] using the culture method indicated that the contamination rates of contact lens and lens storage cases by *Acanthamoeba* were 10.6% and 13.5%, respectively and were considered to be much higher than those reported in the United Kingdom and New Zealand[18,19]. To date, there is a lack of studies performed on the genotyping and physiological characteristics of this free-living amoeba in Malaysia. Consequently, there is currently no molecular epidemiological information regarding *Acanthamoeba* among contact lens wearers in this country. Therefore, this study aimed to determine morphological and molecular characterization of *Acanthamoeba* isolates derived from contact lens paraphernalia using culture enrichment method, polymerase chain reaction (PCR) assay and sequence analysis of the *18S* rRNA gene. Additionally, the pathogenic potential of the positive isolates will also be assessed based on temperature-tolerance and osmo-tolerance assays.

2. Materials and methods

2.1. Collection of contact lens paraphernalia samples

Discarded contact lens paraphernalia (consisting of contact lens, lens storage cases and lens solutions) were donated by 180 contact lens wearers in Peninsular Malaysia from April 2018 to March 2019. The samples were placed individually in zip-locked plastic bags and sent to the Integrative Pharmacogenomics Institute, Universiti Teknologi MARA, Malaysia for the identification of *Acanthamoeba* genus. Individuals who agreed to participate in this study were required to sign an informed consent form according to the guidelines established in the Declaration of Helsinki and Malaysian Guideline for Good Clinical Practice. Additionally, the ethics approval for this study was granted by the Research Ethics Committee of Universiti Teknologi MARA, with a protocol reference number, REC/296/17. In the consent form, participants were given the choice to be informed about positive sample results by providing their e-mail addresses. After approximately one year, the study participants with positive results were contacted through e-mail and questioned about their eye health.

2.2. Isolation and morphological characterization of *Acanthamoeba*

Four non-nutritive agar (NNA) plates (1.5%) (Sigma Aldrich A7002, USA) containing Page's amoeba saline (PAS) (pH 6.9) solution coated with UV inactivated *Escherichia (E.) coli* were prepared for each sample. The lens storage cases were opened under aseptic conditions and the lenses were inoculated onto the first

NNA plate in an upside-down position to ensure the inner part of the lens touched the surface of the agar. In the storage cases containing the lens solution, samples solutions were transferred into sterile tubes, centrifuged for 10 min at 1 500 × *g* and pellets were seeded onto second NNA plate. Next, the storage cases were wiped with a sterile cotton swab soaked with PAS solution to scrape over the internal surface of the contact lens storage cases and directly placed onto the third NNA plate. A new sterile cotton swab was used to scrape over the external surface of the storage cases. The swab was placed into a 2 mL PAS solution and centrifuged for 5 min. This step was performed to ensure the detachment of any remaining organisms on the swab. Subsequently, the pellets were dispensed onto the fourth NNA plate.

Plates inoculated with contact lens, lens solutions and lens storage cases were sealed with Parafilm® and inversely incubated at 30 ℃ with a relative humidity of 85% for 14 d and monitored daily by examination under bright field microscope. Samples were identified as negative if they were found to be deficient in amoeba morphological features within three weeks. If the morphological features were detected, amoeba clones were produced using the migration method[20]. In addition, a block of agar (size 1 cm^2) containing of a minimal number of amoeba was transferred to new NNA plates coated with UV inactivated *E. coli* for at least 1 to 3 times to prevent fungal contamination. The transfer times were determined based on the assessment of fungal growth. The clones were then incubated at 30 ℃ for 2 to 4 d until required for further assays. In addition, no antibiotics were used during the isolation or for further culturing of the isolates.

The morphological characterization of *Acanthamoeba* was performed by harvesting the cysts and/or trophozoites from the NNA plates. The isolates were re-suspended in 0.7% Trypan blue solution and examined under a phase contrast microscope for amoeba viability and the presence of *Acanthamoeba* acanthopodia. The cysts were measured using an ocular micrometre under a light optical microscope and the morphological characteristics were classified according to the respective groups (Ⅰ, Ⅱ and Ⅲ) based on the cyst size and shape, as previously described[4].

2.3. DNA extraction and PCR amplification assay

Acanthamoeba cysts were harvested from the plate on the fifth day of incubation by rinsing the agar surfaces with sterile PAS solution and adjusting the cyst count to 10^4 cysts/mL by serial dilution using a Neubauer counting chamber. Total DNA was extracted using the QIAamp® DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA yield and purity were determined using the NanoDrop® 2000c spectrophotometer (Thermo Fisher Scientific, USA). The DNA was preserved as an aqueous solution at -20 ℃ until further use.

The extracted DNA was subjected to PCR amplification to target a specific recognition sequence of *18S* rRNA amoeba genes from the genus *Acanthamoeba* using genus-specific primers, JDP1 (5'-GGC CCA GAT CGT TTA CCG TGA A-3') and JDP2 (5'-TCT CAC AAG CTG CTA GGG GAG TCA-3'), which amplify a region of ASA.S1 (*Acanthamoeba*-specific amplimer) with an approximate size of 450 base pair (bp)[21]. Amplifications were performed in a 50 μL volume containing 1 μL of DNA template (50 ng/μL), 25 μL of TopTaq Master Mix (2×) (Qiagen, USA), 2 μL of 10 μM oligonucleotide primers for each JDP1 and JDP2 and 20 μL of DNase-free deionized water using DNA thermal cycler (T100™ Bio-Rad Thermal Cycler). The cycling conditions were set up as follows: pre-denaturation step at 94 ℃ for 3 min and 35 cycles of denaturation at 94 ℃ for 1 min, 57 ℃ of annealing for 1 min and then 72 ℃ for 1 min, followed by a final elongation step of 72 ℃ for 10 min. In the control experiments, DNA obtained from a reference strain *Acanthamoeba (A.) castellanii* (ATCC 50492) was used as a positive control and distilled water (instead of DNA) was added to the reaction mixture as a negative control. A 100 bp ladder was used as a DNA size marker (Biolabs, USA). Aliquots of 10 μL from each PCR reaction was subjected to a 1.5% agarose gel electrophoresis, stained with 0.5 μg/mL ethidium bromide and observed under a UV illumination chamber (UVP, Cambridge, United Kingdom).

2.4. Sequence alignment and phylogenetic analysis

The PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Germany) according to the manufacturer's instructions. The sequencing was performed in an ABI PRISM BigDye® Terminator v.3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) based on the standard Sanger Dideoxynucleotide method and sequenced in both directions. The sequences were edited and aligned using the MEGA 6.0 software program. The sequences obtained were compared by the Basic Local Alignment Search Tool program of the US National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) to classify current *Acanthamoeba* isolates into different species. In addition, the nucleotide sequences of all the *Acanthamoeba* genotypes were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/pubmed). The phylogenetic tree was reconstructed using Kimura-two-parameter distance algorithm with 1 000 replicates using neighbor-joining method. The sequence of the *Hartmanella* sp. (NCBI KF697197) strain was used to root the final phylogenetic tree.

2.5. Temperature-tolerance and osmo-tolerance assays

Temperature-tolerance and osmo-tolerance assays were performed as previously described[8]. For the temperature-tolerance assay, *Acanthamoeba* cysts were harvested from plates and counted using a hemacytometer. Then, approximately 10^3 cysts were inoculated

in the centre position of different NNA plates coated with UV inactivated *E. coli* (strain K-12, American Type Culture Collection, Manassas, VA). The plates were incubated at 37 ℃ and 42 ℃, respectively, and examined for amoebic growth with a bright field microscope for up to 5 d. For the investigation of osmo-tolerance, approximately 10^3 cysts were inoculated onto 1.5% NNA plates containing mannitol 0.5 M and 1.0 M, respectively, and coated with UV inactivated *E. coli*. The plates were incubated at 30 ℃ and monitored using a bright field microscope at 24, 48, 72, 96 and 120 h. NNA plates without the addition of mannitol were used as a negative control.

For the evaluation of any evidence of growth or absence of growth during this stage, the number of cysts or trophozoites detected approximately 20 mm from the centre of each plate was calculated and scored based on the following: 0 (-), 1-15 (+), 16-30 and (++) and >30 (+++)[22]. All experiments were performed in triplicates and mean values were calculated from individual tests. For cases with plus scoring, the absolute mean values were recorded as a round figure. The strain *A. castellanii* (ATCC 50492) was selected as a reference strain for a potentially pathogenic isolate.

3. Results

3.1. Acanthamoeba isolation, growth and morphological characterization

In this study, the presence of *Acanthamoeba* in NNA cultures was detected in 7.8% (14/180) of contact lens paraphernalia samples consisting of lens storage cases (n=7), lens solutions (n=4) and contact lens (n=3). The amoeba isolates from contact lens paraphernalia demonstrated phenotypic differences, whereby 78.6% (11/14) was classified as group Ⅱ which consisted of a two-layer membrane with the outer wall moderately undulated (exocyst) and an endocyst wall typically a polygonal arrangement that was connected at the pore zone. The remaining 21.4% (3/14) belonged to group Ⅲ species which typically exhibited a thin, smooth ectocyst closely appose to round endocyst and smaller in size (Figure 1).

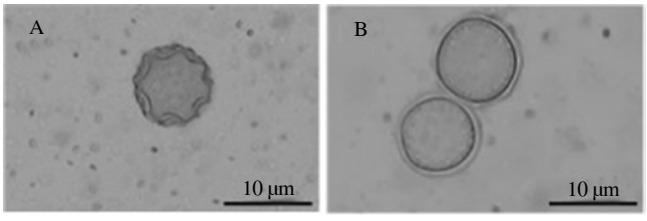

Figure 1. Predominant cyst morphotypes observed in five day old culture. A. Morphotype Ⅱ observed in 11 isolates. B. Morphotype Ⅲ in isolates CL5, CL171 and CL176. Light microscope ×400. Scale bar: 10 μm (original).

3.2. Molecular characterization of Acanthamoeba species

Acanthamoeba was confirmed in all positive-culture samples as demonstrated by the amplification of the ASA.S1 region using a genus-specific primer pair (Figure 2). The sequence homology search results for these 14 *Acanthamoeba* species using the NCBI databank revealed homology with the *Acanthamoeba* spp. strains presented in Table 1. The phylogenetic tree reconstructions based on the neighbor-joining method yielded similar topologies, thereby placing the 14 *Acanthamoeba* species examined in this study within the genotype T4 clade (Figure 3). The results revealed that CL5, CL118 and CL171 showed 99%-100% similarity with the previously identified strain IT-N (KF881887); CL14, CL54 and CL114 showed 98%-99% similarity with strain IK-WW74 (LC373015); CL45 and CL126 showed 100% similarity with strain OSU-07-019 (MN239986); CL107 showed 100% similarity with strain SA3-SLG3 (MH790982); CL149 showed 97% similarity with strain UTA4-HT14 (MH790988); CL172 and CL177 showed 99%-100% similarity with strain Ubo19 (KF733262); CL173 showed 100% similarity with strain IK-HD177 (LC276839) and CL176 showed 99% similarity with strain BTGA5-BTG25 (MH790997). Among the 14 *Acanthamoeba* spp. isolates, seven isolates were not 100% identical to any of the strains available in the GenBank database, thereby indicating that certain polymorphisms can exist within the nucleotide sequences. The sequences of the ASA.S1 amplicons obtained in this study were deposited in the GenBank database under the accession numbers MH791017-MH791025 and MN700306-MN700310, respectively.

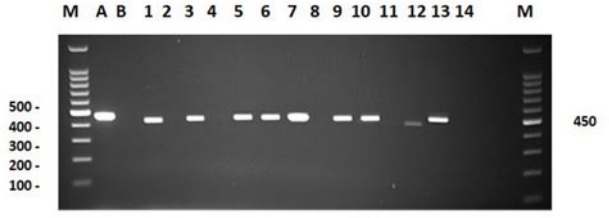

Figure 2. Representative photograph of 1.5% agarose gel showing the amplified product of 450 bp region (ASA.S1) of the *18S* rRNA gene of *Acanthamoeba* spp. Lane A: positive control (*A. castellanii* ATCC 50492); Lane B: negative control (distilled water); Lanes 1-14: positive isolates; Lane M: DNA ladder (100 bp).

3.3. Pathogenic potential of Acanthamoeba

All the samples investigated in this study revealed that *Acanthamoeba* strains were able to grow at 35 ℃, similar to the temperature of the human eye. Additionally, 13 (92.9%) and 9 (64.3%) isolates were able to grow at temperatures of 37 ℃ and 42 ℃, respectively. For the osmo-tolerance test, all isolates were able to grow in 0.5 M mannitol, while only 9 (64.3%) isolates were able to grow in 1 M mannitol (Table 2). Thus, these nine isolates

Table 1. Comparison of *Acanthamoeba* species isolates obtained from 14 contact lens paraphernalia positive samples with reference strains available in the GenBank database.

Isolates	Accession number	Genotype	Strain with highest homology	Accession number	Region of origin
CL5	MH791017	T4	lt-N	KF881887	Taiwan
CL14	MH791018	T4	IK-WW74	LC373015	Taiwan
CL45	MH791019	T4	OSU-07-019	MN239986	USA
CL54	MH791020	T4	IK-WW74	LC373015	Taiwan
CL107	MH791021	T4	SA3-SLG3	MH790982	Malaysia
CL114	MH791022	T4	IK-WW74	LC373015	Taiwan
CL118	MH791023	T4	lt-N	KF881887	Taiwan
CL126	MH791024	T4	OSU-07-019	MN239986	USA
CL149	MH791025	T4	UTA4-HT14	MH790988	Malaysia
CL171	MN700306	T4	lt-N	KF881887	Taiwan
CL172	MN700307	T4	Ubol9	KF733262	Thailand
CL173	MN700308	T4	IK-HD177	LC276839	Iran
CL176	MN700309	T4	BTGA5-BTG25	MH790997	Malaysia
CL177	MN700310	T4	Ubol9	KF733262	Thailand

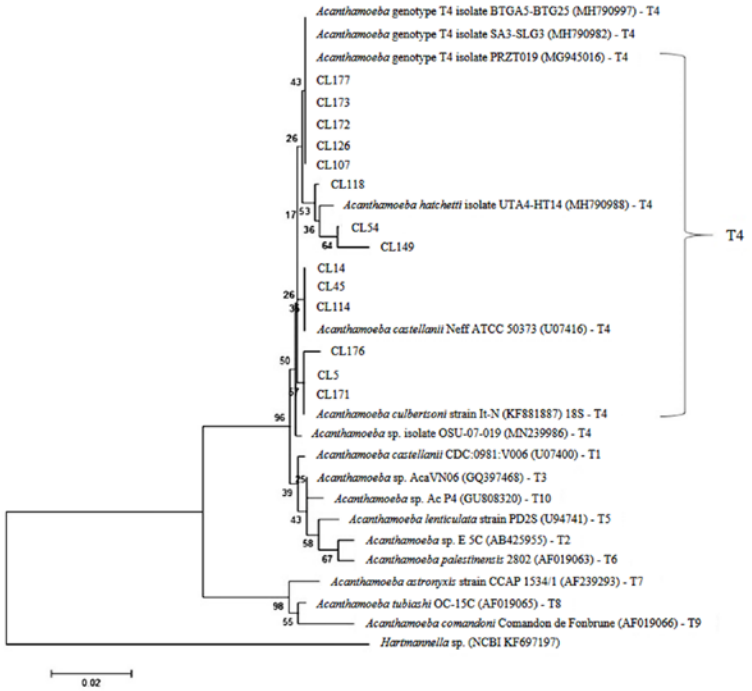

Figure 3. Phylogenetic tree reconstructed using Kimura two-parameter distance algorithm with 1 000 replicates using neighbor-joining method. The sequence of *Hartmanella* sp. (NCBI KF697197) was used to root the final phylogenetic tree.

Table 2. Results of the study on the growth of *Acanthamoeba* isolates at different temperatures (37 ℃ or 42 ℃) and osmolarities (0.5 M or 1 M).

Isolates	Morphological group	Genotype	Temperature-tolerance		Osmo-tolerance	
			37 ℃	42 ℃	0.5 M mannitol	1 M mannitol
CL5	III	T4	+++	+	+++	+++
CL14	II	T4	+++	+	+++	+++
CL45	II	T4	+++	-	+++	-
CL54	II	T4	+++	+++	+++	+++
CL107	II	T4	+++	-	+++	-
CL114	II	T4	+++	+	+++	+++
CL118	II	T4	+++	++	+++	+++
CL126	II	T4	+++	++	+++	+++
CL149	II	T4	+++	+	+++	+++
CL171	III	T4	+++	+	+++	+++
CL172	II	T4	+++	+	+++	+++
CL173	II	T4	+++	-	+++	-
CL176	III	T4	+++	-	+++	-
CL177	II	T4	-	-	+++	-
Reference strain *A. castellanii* (ATCC 50492)	II	T4	+++	++	+++	++

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

(CL5, CL14, CL54, CL114, CL118, CL126, CL149, CL171 and CL172) were considered to be potentially pathogenic, displaying good or excellent growth at 37 ℃ (temperature-tolerance) and in 1 M mannitol (osmo-tolerance). The only strain that displayed an excellent (+++) score at 37 ℃, 42 ℃, 0.5 M and 1 M mannitol was isolate CL54. Therefore, all the isolates in this study were classified as members of the group Ⅱ, group Ⅲ and genotype T4. The reference strain used in this study, (*A. castellanii*, ATCC 50492) also survived the growth conditions at 42 ℃ and 1 M mannitol although the cell count was lower as compared to its growth at 37 ℃ and 0.5 M mannitol.

4. Discussion

Contact lenses are a safe and cheap cosmetic alternative that can also provide effective vision correction. However, contact lens wearers are at risk of contracting *Acanthamoeba* keratitis if they fail to follow the recommended procedures for cleaning, disinfection and storing their contact lens[23]. This observation has been validated in a few studies reporting that more than 90% of *Acanthamoeba* keratitis cases occur in contact lens wearers[15,24]. In this study, *Acanthamoeba* was detected in 14 (7.8%) out of 180 contact lens paraphernalia samples investigated using cell culture and PCR-based methods. Interestingly, these results indicate a high prevalence of *Acanthamoeba* as compared to the 6.7% from lens storage cases among 150 students in the Nakhonpathom Province, Thailand[25]. In another study based in Hong Kong, China, lens storage cases analysed using conventional PCR demonstrated a low contamination rate of 1%[26]. The higher prevalence rate observed in this study concurs with a previous local study that reported a rate of 8% (14/175). These observations are particularly worrying and may present a potential reservoir for many more cases of infection. Additionally, the highest *Acanthamoeba* isolation at 50% (7/14) was derived from the storage cases, similar to other studies reported worldwide[27,28]. Most cases of contact lens-associated *Acanthamoeba* keratitis can be attributed to some degree of negligence in following the recommended lens hygiene procedures. For instance, the lens storage cases should be thoroughly cleaned once a week using mild detergent and a soft-bristol brush, followed by rinsing with sterile saline solution and left to dry. The storage case should also be replaced periodically as recommended by Mahittikorn *et al*[29]. It is envisaged that these recommendations could also reduce the incidence of the more commonly occurring bacterial and fungal keratitis infections associated with contact lens.

The morphological determination was rather difficult to achieve in some cases as the cysts, although derived from one single clone, appeared to have varied morphologies due to intraspecific polymorphism being quite common among *Acanthamoeba*. Nonetheless, among the fourteen *Acanthamoeba* isolates obtained from contact lens paraphernalia, 11 isolates (78.6%) were classified as group Ⅱ, while the rest (21.4%) exhibited group Ⅲ morphology based on the morphological features of their cystic forms[4]. This finding was consistent with previous report indicating that group Ⅱ typically harbours most of the pathogenic *Acanthamoeba* clinical isolates[30]. Although most of the pathogenic species of *Acanthamoeba* reside in group Ⅱ (*A. castellanii* and *A. hatchetti*), one species in group Ⅲ (*A. culbertsoni*) has also been reported as causative agent of keratitis in some cases[5].

It is important to note that the size of amplicons used in the phylogenetic analyses of the *18S* rRNA gene sequence is essential to obtain a positive amoeba isolate identification. In this genotypic study, the ASA.S1 fragment was used for the identification of *Acanthamoeba*-positive isolates, in which all of them were found to belong to the genotype T4. This finding is consistent with a recent study conducted in Argentina, whereby 100% of the isolates were also identified as genotype T4[31]. In addition, another study also showed evidence that ten clinical isolates of *Acanthamoeba* from patients with *Acanthamoeba* keratitis in Malaysia, 100% were linked to genotype T4[32]. These findings point to the fact that this genotype is regarded as the most common and predominant genotype amongst all the known genotypes (T1-T21) found in corneal infections worldwide[33,34]. The *Acanthamoeba* genotype T4 is thought to display some form of evolutionary lineage-associated *Acanthamoeba* keratitis and have the capability of creating an immunosuppressive behaviour by stimulating the release of proinflammatory cytokines and the early production of IL-10 in innate immune cells. These features may promote the immune evasion of *Acanthamoeba* by inducing a limited inflammatory response *in situ*. Hence, together with the increasing evidence indicating that the vast majority of environmental, keratitis and non-keratitis *Acanthamoeba* isolates belong to genotype T4, the pathogenicity of this genotype is further reinforced[7,31].

In this study, the strains isolated from the contact lens paraphernalia were identified as *A. castellanii, A. culbertsoni, A. hatchetti* and genotype T4. *A. castellanii, A. culbertsoni* and genotype T4 are commonly found in natural environments and considered to be pathogenic[35]. This finding also corresponds well to the fact that the majority of the keratitis-causing *Acanthamoeba* from the UK, Iran and Korea are *A. castellanii* and genotype T4[36,37]. Moreover, four corneal scraping samples obtained from patients of the Farabi Eye Reference Center, Iran were identified as *A. culbertsoni* and genotype T4, which are the predominant genotype in Iran[38]. Nevertheless, *A. hatchetti* can also be of clinical relevance, as they have been isolated from contact lens-wearing keratitis patients in Austria[39]. It is interesting to note that this study demonstrates the *A. hatchetti* in contact lens paraphernalia in Malaysia.

Several *Acanthamoeba* strains from diverse genotypes can cause

human infections, but different isolates within one genotype can possess different pathogenic potentials. Temperature-tolerance and osmo-tolerance behaviours are widely accepted as a prerequisite for pathogenicity assessment in *Acanthamoeba*[20]. For instance, *Acanthamoeba* strains that infect for humans (and other mammals) must be able to survive at 37 ℃ as well as higher body temperatures[40]. The results obtained in this study revealed that 9 (64.3%) *Acanthamoeba* isolates from contact lens paraphernalia displayed good or excellent growth at 37 ℃ or higher temperatures and were able to grow at concentrations of 0.5 M mannitol or higher. This observation is in line with the previous studies which have also shown to possess temperature-tolerance and osmo-tolerance attributes[5,41]. However, further investigations should be performed with the isolated strains to assess if the temperature-tolerance and osmo-tolerance assays can be utilised as an indicator of the pathogenic potential for non-clinical and environmental *Acanthamoeba* strains. Besides, it is evident that other factors such as the prolonged culture cycles for some of the strains could also result in the loss of ability to grow in high temperature and osmotic pressure conditions as previously demonstrated by other researchers[5,42].

To the best of our knowledge, this is the first study on the classification of *Acanthamoeba* strains at the genotype level and its pathogenic potential among contact lens wearers in Malaysia. The finding in this study substantiates the observation that among all of the *Acanthamoeba* genotypes reported to date, T4 appears to be the most prevalent and predominant genotype identified as a typical causative agent of *Acanthamoeba* keratitis. The presence of potentially pathogenic T4 genotype at elevated temperature and higher osmolarity could pose a high risk for people using contact lens. Hence, increasing awareness efforts should be promoted by the public health authorities in this country for the prevention of contamination by *Acanthamoeba* and proper disinfection procedures of contact lens paraphernalia among contact lens wearers.

Conflict of interest statement

The authors declare they have no conflict of interest.

Funding

This study was partly supported by the Integrative Pharmacogenomics Institute (241910/2017/TSA/13) and Geran Inisiatif Penyeliaan [600-IRMI 5/3/GIP (024/2019)], Universiti Teknologi MARA, Malaysia. The funding agency had no role in the study design, data collection and interpretation.

Authors' contributions

The concept study was conducted and designed by TSA, MKAG, NAK and RS. RHMH, NSMI and KAK performed the samples collection and laboratory experiments. TSA, MKAG and RHMH were responsible for the analysis and interpretation of the data. TSA and RHMH wrote and drafted the manuscript. NAK, RS, MKAG, NSMI and KAK reviewed and edited the manuscript, and then gave final approval of the version to be published. All author read and approved the final manuscript.

References

[1] Marciano-Cabral F, Cabral G. *Acanthamoeba* spp. as agents of disease in humans. *Clin Microbiol Rev* 2003; **16**(2): 273-307.

[2] Illingworth CD, Cook SD. *Acanthamoeba* keratitis. *Surv Ophthalmol* 1998; **42**(6): 493-508.

[3] Walochnik J, Aichelburg A, Assadian O, Steuer A, Visvesvara G, Vetter N, et al. Granulomatous amoebic encephalitis caused by *Acanthamoeba amoebae* of genotype T2 in a human immunodeficiency virus-negative patient. *J Clin Microbiol* 2008; **46**(1): 338-340.

[4] Pussard M, Pons R. Morphology of cystic wall and taxonomy of genus *Acanthamoeba* (Protozoa, Amoebidae). *Protistologica* 1977; **8**: 557-598.

[5] Lorenzo-Morales J, Khan N, Walochnik J. An update on *Acanthamoeba* keratitis: Diagnosis, pathogenesis and treatment. *Parasite* 2015; **22**: 10.

[6] Fuerst PA, Booton GC, Crary M. Phylogenetic analysis and the evolution of the *18S* rRNA gene typing system of *Acanthamoeba*. *J Eukaryot Microbiol* 2015; **62**(1): 69-84.

[7] Booton GC, Visvesvara GS, Byers TJ, Kelly DJ, Fuerst PA. Identification and distribution of *Acanthamoeba* species genotypes associated with nonkeratitis infections. *J Clin Microbiol* 2005; **43**(4): 1689-1693.

[8] Mohd Hussain RH, Ishak AR, Abdul Ghani MK, Ahmed Khan N, Siddiqui R, Shahrul Anuar T. Occurrence and molecular characterization of *Acanthamoeba* isolated from recreational hot springs in Malaysia: Evidence of pathogenic potential. *J Water Health* 2019; **17**(5): 813-825.

[9] Stapleton F, Keay L, Edwards K, Naduvilath T, Dart JK, Brian G, et al. The incidence of contact lens-related microbial keratitis in Australia. *Ophthalmology* 2008; **115**(10): 1655-1662.

[10] Qvarnstrom Y, Visvesvara GS, Sriram R, da Silva AJ. Multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris* and *Naegleria fowleri*. *J Clin Microbiol* 2006; **44**(10): 3589-3595.

[11] Moriyama AS, Hofling-Lima AL. Contact lens-associated microbial keratitis. *Arquivos Brasileiros de Oftalmologia* 2008; **71**(1): 32-36.

[12] Jeong HJ, Yu HS. The role of domestic tap water in *Acanthamoeba* contamination in contact lens storage cases in Korea. *Korean J Parasitol* 2005; **43**(2): 47-50.

[13] Pens CJ, da Costa M, Fadanelli C, Caumo K, Rott M. *Acanthamoeba*

spp. and bacterial contamination in contact lens storage cases and the relationship to user profiles. *Parasitol Res* 2008; **103**(6): 1241-1245.

[14]Niyyati M, Rahimi F, Lasejerdi Z, Rezaeian M. Potentially pathogenic free-living amoebae in contact lenses of the asymptomatic contact lens wearers. *Iran J Parasitol* 2014; **9**(1): 14-19.

[15]Martin-Navarro CM, Lorenzo-Morales J, Cabrera-Serra MG, Rancel F, Coronado-Alvarez NM, Pinero JE, et al. The potential pathogenicity of chlorhexidine-sensitive *Acanthamoeba* strains isolated from contact lens cases from asymptomatic individuals in Tenerife, Canary Islands, Spain. *J Med Microbiol* 2008; **57**(11): 1399-1404.

[16]Kamel AG, Norazah A. First case of *Acanthamoeba* keratitis in Malaysia. *Trans R Soc Trop Med Hyg* 1995; **89**(6): 652.

[17]Mutalib HA, Majid SA, Ghani MKA, Nordin A, Suboh Y, Moktar N. Causes of *Acanthamoeba* spp. infection among contact lens wearers in Kuala Lumpur. *Malays J Health Sci* 2005; **3**(2): 9-17.

[18]Larkin DF, Kilvington S, Easty DL. Contamination of contact lens storage cases by *Acanthamoeba* and bacteria. *Br J Ophthalmol* 1990; **74**(3): 133-135.

[19]Gray TB, Cursons RT, Sherwan JF, Rose PR. *Acanthamoeba*, bacterial and fungal contamination of contact lens storage cases. *Br J Ophthalmol* 1995; **79**(6): 601-605.

[20]Gianinazzi C, Schild M, Wuthrich F, Muller N, Schurch N, Gottstein B. Potentially human pathogenic *Acanthamoeba* isolated from a heated indoor swimming pool in Switzerland. *Exp Parasitol* 2009; **121**(2): 180-186.

[21]Garcia LS. *Clinical microbiology procedures handbook*. 3rd edition. Washington DC: ASM Press; 2010.

[22]Landell MF, Salton J, Caumo K, Broetto L, Rott MB. Isolation and genotyping of free-living environmental isolates of *Acanthamoeba* spp. from bromeliads in Southern Brazil. *Exp Parasitol* 2013; **134**(3): 290-294.

[23]Carnt N, Stapleton F. Strategies for the prevention of contact lens-related *Acanthamoeba* keratitis: A review. *Ophthalmic Physiol Opt* 2016; **36**(2): 77-92.

[24]Sengor T, Kurna SA, Altun A, Irkec M, Aki SF, Aksoy S. Contact lens-related *Acanthamoeba* keratitis and accompanying dacryoadenitis. *Eye Contact Lens* 2015; **41**(4): 204-209.

[25]Roongruangchai K, Supadirekkul P. Contamination of contact lens cases by *Acanthamoeba* in Thailand. *J Trop Med Parasitol* 1997; **20**: 25-29.

[26]Boost M, Cho P, Lai S, Sun WM. Detection of *Acanthamoeba* in tap water and contact lens cases using polymerase chain reaction. *Optom Vis Sci* 2008; **85**(7): 526-530.

[27]Schroeder JM, Booton GC, Hay J, Niszl IA, Seal DV, Markus MB, et al. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba* from humans with keratitis and from sewage sludge. *J Clin Microbiol* 2001; **39**(5): 1903-1911.

[28]Rivera W, Edric DV. 18S ribosomal DNA genotypes of *Acanthamoeba* species isolated from contact lens cases in the Philippines. *Parasitol Res* 2009; **105**(4): 1119-1124.

[29]Mahittikorn A, Kittichathanakul T, To-Im J, Nacapunchai D. Knowledge, behavior and free-living amoebae contamination of cosmetic contact lens among university wearers in Thailand: A cross-sectional study. *Eye Contact Lens* 2017; **43**(2): 81-88.

[30]Risler A, Coupat-Goutaland B, Pelandakis M. Genotyping and phylogenetic analysis of *Acanthamoeba* isolates associated with keratitis. *Parasitol Res* 2013; **112**(11): 3807-3816.

[31]Casero RD, Mongi F, Laconte L, Rivero F, Sastre D, Teheran A, et al. Molecular and morphological characterization of *Acanthamoeba* isolated from corneal scrapes and contact lens wearers in Argentina. *Infect Genet Evol* 2017; **54**: 170-175.

[32]Ng SL, Nordin A, Ghafar NA, Suboh Y, Rahim NA, Chua KH. *Acanthamoeba*-mediated cytopathic effect correlates with MBP and AhLBP mRNA expression. *Parasit Vectors* 2017; **10**(1): 625.

[33]Rahdar M, Niyyati M, Salehi M, Feghhi M, Makvandi M, Pourmehdi M, et al. Isolation and genotyping of *Acanthamoeba* strains from environmental sources in Ahvaz City, Khuzestan province, southern Iran. *Iran J Parasitol* 2012; **7**(4): 22-26.

[34]Mirjalali H, Niyyati M, Abedkhojasteh H, Babaei Z, Sharifdini M, Rezaeian M. Pathogenic assays of *Acanthamoeba* belonging to the T4 genotype. *Iran J Parasitol* 2013; **8**(4): 530-535.

[35]Kao PM, Hsu BM, Chen CT, Huang SW, Kao ES, Chen JL, et al. Identification and quantification of the *Acanthamoeba* species and genotypes from reservoirs in Taiwan by molecular techniques. *Acta Trop* 2014; **132**: 45-50.

[36]Kong HH, Shin JY, Yu HS, Kim J, Hahn TW, Chung DI. Mitochondrial DNA restriction fragment length polymorphism (RFLP) and 18S small-subunit ribosomal DNA PCR-RFLP analyses of *Acanthamoeba* isolated from contact lens storage cases of residents in Southwestern Korea. *J Clin Microbiol* 2002; **40**(4): 1199-1206.

[37]Maghsood AH, Sissons J, Rezaian M, Nolder D, Warhurst D, Khan NA. *Acanthamoeba* genotype T4 from the UK and Iran and isolation of the T2 genotype from clinical isolates. *J Med Microbiol* 2005; **54**(8): 755-759.

[38]Ghamilouie MM, Valadkhani Z, Khoshzaban F. Species identification of *Acanthamoeba* strains isolated from patients referring to Farabi Eye Reference Center using PCR-RFLP method. *J Med Microbiol Infec Dis* 2014; **2**(3): 125-129.

[39]Walochnik J, Haller-Schober EM, Kölli H, Picher O, Obwaller A, Aspock H, et al. Discrimination between clinically relevant and nonrelevant *Acanthamoeba* strains isolated from contact lens-wearing keratitis patients in Austria. *J Clin Microbiol* 2000; **38**(11): 3932-3936.

[40]Schuster FL, Visvesvara GS. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *Int J Parasitol* 2004; **34**(9): 1001-1027.

[41]Wagner C, Reyes-Batlle M, Ysea MA, Perez MV, de Rondon CG, Paduani AJ, et al. Genotyping of clinical isolates of *Acanthamoeba* genus in Venezuela. *Acta Parasitol* 2016; **61**(4): 796-801.

[42]Kohsler M, Leitsch D, Furnkranz U, Duchene M, Aspock H, Walochnik J. *Acanthamoeba* strains lose their abilities to encyst synchronously upon prolonged axenic culture. *Parasitol Res* 2008; **102**(5): 1069-1072.