ISOLATION AND IDENTIFICATION OF *ACANTHAMOEBA* SPP FROM WATER AND SOIL IN SOUTHERN THAILAND

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Abstract. *Acanthamoeba* spp are free-living amoebae commonly found in a wide range of environment settings and can cause granulomatous amoebic encephalitis and *Acanthamoeba* keratitis. Here, we collected water (n = 51) and soil (n = 20) samples from areas associated with human activities in southern Thailand. Free-living amoebae were found in 56 samples, 56% morphologically identified as *Acanthamoeba* spp, among which, based on cyst morphology, 15, 50 and 35% were placed in morphological group I, II and III, respectively. Based on 18S rDNA gene sequences, 19 isolates were confirmed as *Acanthamoeba* spp, among which 6, 3, 1, 1, 2 and 2 isolates belonged to genotype T4, T5, T11, T17, T18, and T20, respectively. *Acanthamoeba* spp in morphological groups II and III and genotypes T4, T5, T11, and T18 are believed to be associated with serious human infection. This is the first report of the environmental presence of clinically pathogenic *Acanthamoeba* spp in southern Thailand.

Keywords: *Acanthamoeba* spp, environment source, free-living amoeba, pathogen, southern Thailand

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INTRODUCTION

Acanthamoeba spp are free-living amoebae (FLA), which are both opportunistic and pathogenic, and widely distributed in a variety of environmental settings, such as contact lens solution, chlorinated swimming pool, dust, freshwater, seawater, and soil (Siddiqui and Khan, 2012; Tanveer *et al*, 2013). A sizeable number of *Acanthamoeba* spp pose a serious

health risk to humans and animals (Winck *et al*, 2011). Granulomatous amoebic encephalitis (GAE) and skin infections due to *Acanthamoeba* spp have been reported in immunocompromised patients (Khan, 2006; Lackner *et al*, 2010). In addition, *Acanthamoeba* spp are responsible for *Acanthamoeba* keratitis (AK) in contact lens wearers, a condition that can lead to scarring of the cornea and resulting in permanent visual impairment or complete blindness (Li *et al*, 2019; Carnt *et al*, 2020).

In Thailand, a retrospective review of the medical records of seven patients with AK, treated at Ramathibodi Hospital, Mahidol University, Bangkok between 1998 and 2008, revealed an association with the wearing of soft contact lens and washing eyes with tap water (Chuckpaiwong *et al*, 2010). On the other hand, a review of AK patients treated at Siriraj Hospital, Mahidol University, Bangkok during 1996-2006 found AK can occur in any persons (Wanachiwanawin *et al*, 2012). The first case in Thailand of AK in an immunocompetent patient without a history of contact lens use was recently reported (Tananuvat *et al*, 2019). Other important risk factors of AK in Thailand are swimming in a dam and diving in a natural pond (Bunsuwansakul *et al*, 2019).

Agricultural workers come in regular contact with water and soil, which serve as sources of organisms that disseminate diseases to humans and animals. In Thailand *Acanthamoeba* spp were detected in a number of environmental situations, with 13 and 15.9% of samples from hot springs in 13 provinces in central and southern regions (Lekkla *et al*, 2005) and natural water sources from 11 provinces in northeast region (Thammaratana *et al*, 2016) respectively. Potentially pathogenic FLA was also detected during the 2011 flooding of Chiang Mai, located in the north of the country (Wannasan *et al*, 2013).

Identification of *Acanthamoeba* spp by a plate culture technique is the classical gold standard (Schuster, 2002). Trophozoites of *Acanthamoeba* spp can be recognized by their needle-like projections and are classified based on cyst size and shape in three distinct morphological groups, namely, I, II, and III (Pussard *et al*, 1977). As it is not possible to definitively identify an *Acanthamoeba* sp from morphological characteristics, molecular studies based on 18S rDNA gene sequences have allowed identification of 22 genotypes (T1-T22) (Coronado-Velázquez *et al*, 2020).

Information regarding *Acanthamoeba* spp from environmental samples in southern Thailand is lacking. Here, a culture technique was employed to determine the presence of *Acanthamoeba* spp in water and soil samples in the southern region and identity of the isolates confirmed using molecular method. The findings should provide information regarding

presence of isolates with pathological potential so that appropriate control measures can be undertaken in this region of the country.

MATERIALS AND METHODS

Sampling sites and samples collection

Samples were collected during the period May 2019 to February 2020 from residential areas, nearby residential areas and public spaces where human activities, such as agriculture, fishing, and traditional cultural practices, take place. Water samples (n = 51) from ditches (n = 8), marshes (n = 10), canals (n = 14) and wells (n = 19), and soil samples (n = 20) were collected from at different locations in five provinces of southern Thailand (Fig 1). At least 2 liters of water were collected at each site, and soil samples (~200 g each) at a depth of 2-5 cm in areas not exposed to direct sunlight were collected in Narathiwat Province only.

Cultivation of FLA

Water samples were cultured as described by Fabres *et al* (2016) and soil samples as described by Mohaghegh *et al* (2016) with some modification. In brief, a 2 liters of water sample were filtered through a 1.5 μm pore size membrane filter and filtrate, suspended in 20 ml of Page's amoebic saline (PAS) ($MgSO_4.7H_2O$, Na_2HPO_4, NaCl, $CaCl_2.2H_2O$, KH_2PO_4: Merck, Darmstadt, Germany), were centrifuged at 553g for 15 minutes. Pellet was spread onto heat-inactivated *Escherichia coli* pre-coated non-nutrient agar (NNA) medium (Agar no.1: BD Difco, Sparks, MD; $MgSO_4.7H_2O$, Na_2HPO_4, NaCl, $CaCl_2.2H_2O$, KH_2PO_4: Merck, Darmstadt, Germany) plate, sealed with paraffin, and incubated at ambient temperature. For a soil sample, 2 g were suspended in 10 ml of PAS, centrifuged at 553g for 10 min and pellet cultured in NNA as described above. All plates were observed under an inverted microscope (200x magnification) daily for growth of FLA for 7 days. Strains were maintained by placing a small piece of FLA-containing agar in an NNA plate and incubated as described above.

Morphological identification of *Acanthamoeba* spp

A sample of PAS was removed with a sterilized inoculation loop, transferred onto a glass slide in a drop of sterile PAS and examined under a bright-field microscope (400x magnification). FLA trophozoites were observed after incubation for 1-3 days, while cysts were observed after 7 days. FLA was identified as *Acanthamoeba* spp by appearance of trophozoite movement in a needle-like projection. Group I specimen exhibited a large cyst (diameter >18 μm) with smooth or mildly wrinkled outer cell walls; group II specimen exhibited two-layer membranes (diameter <18 μm) with moderately undulating ectocyst and endocyst walls exhibiting a polygonal arrangement connected at

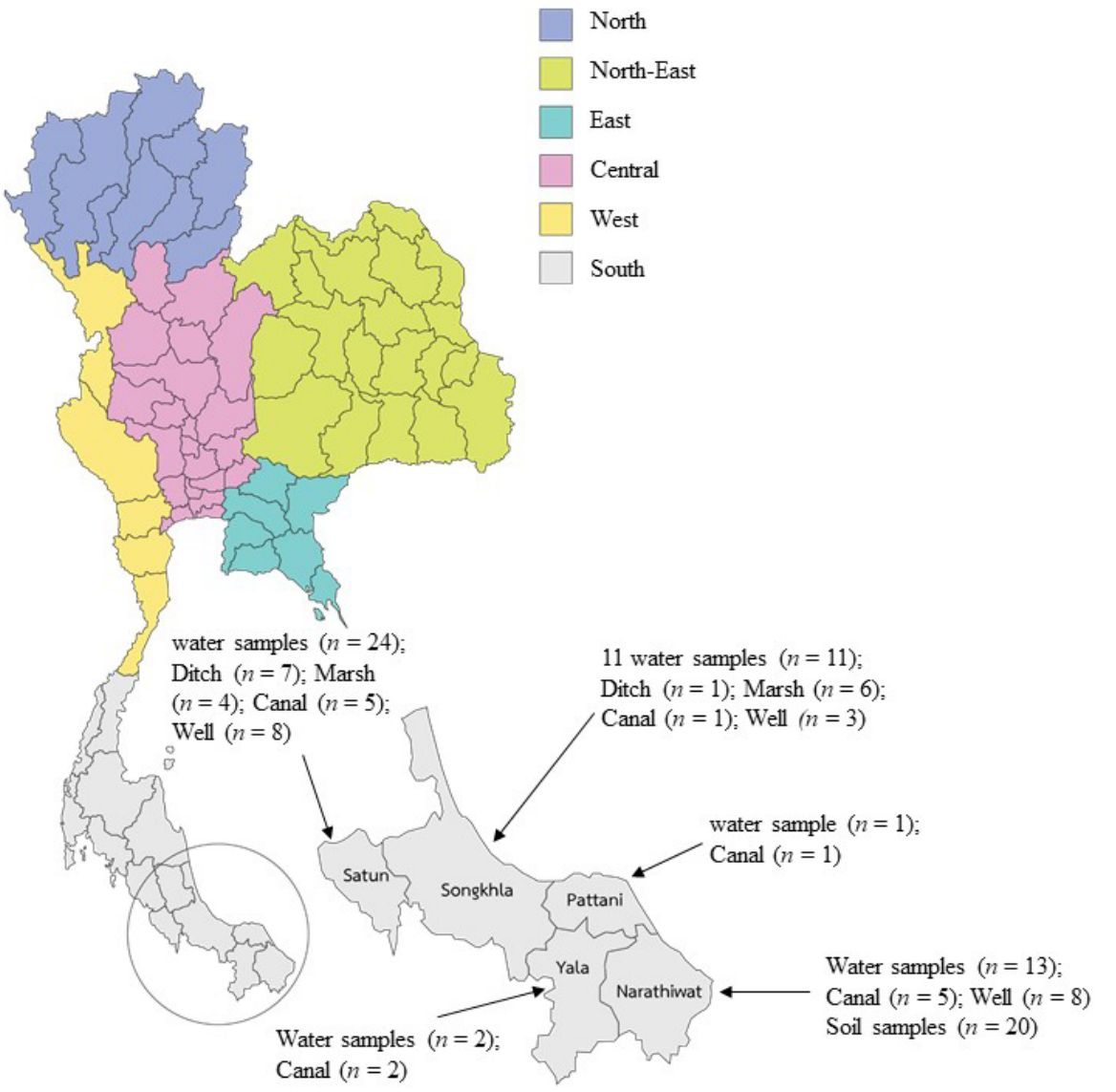

Fig 1 - Map of sampling sites in five provinces (located in circle) of southern Thailand

the pore zone; and group III specimen exhibited a cyst (diameter ≤18 μm) with a thin, smooth ectocyst closely opposed to a round endocyst (Pussard *et al*, 1977).

Molecular identification of *Acanthamoeba* spp

DNA was extracted from culture samples morphologically classified as *Acanthamoeba* spp using a QIAamp DNA Mini extraction kit (Qiagen, Hilden, Germany) and stored at -20°C until used. PCR was performed using *Acanthamoeba*-specific primer pairs, JDP1 (5'-GGCCCAGATCGTTTACCGTGAA-3') and JDP2 (5'-TCTCACAAGCTGCTAGGGAGTCA-3') targeting 18S rDNA (Schroeder *et al*, 2001). Reaction (20 μl) contained 1X TopTaq Master Mix Kit (Qiagen, Hilden, Germany), 10 pmol of each primer, and 100-200 ng of DNA. Thermocycling was carried out in a T100 Thermal Cycler (Applied Biosystem, Foster City, CA) as follows: 94°C for 5 minutes; 40 cycles of 94°C for 60 seconds, 62°C for 60 seconds and 72°C for 60 seconds; with a final step of 72°C for 5 minutes. Amplicon (450 bp) was analyzed by 2% agarose gel-electrophoresis and staining with Safe-GreenTM dye (Applied Biological Materials, Richmond, Canada). Bands were excised and directly sequenced (Macrogen, Seoul, Korea).

Phylogenetic characterization

The 18S rDNA gene sequences were compared to other sequences in the GenBank database using a Basic Local Alignment Search Tool (BLAST) search engine. Multiple alignment and pairwise alignment were performed using Clustal W in a BioEdit program (version 7.0.4.1) (Hall, 1999). Cluster analysis of sequences was performed by constructing a phylogram using program MEGA6 (Tamura *et al*, 2013) and phylogenetic trees were constructed using a neighbor-joining distance (NJ) method with a generation of 1000 bootstrapped replicates. Consensus tree was rooted by Balamuthia mandrillaris (GenBank accession no. AF019071) and the highest percent similarity is accepted for species identification. The 18S rDNA sequences of local *Acanthamoeba* strains (n = 15) were deposited in GenBank database (accession no. MW181627-MW181641).

Statistical analysis

Fisher's Exact test was used to determine significant differences of *Acanthamoeba* spp in water sources from five different provinces in southern Thailand. A p-value <0.05 is considered significant.

RESULTS

Morphological identification of *Acanthamoeba* spp

Sampling of water and soil samples collected from five provinces in southern Thailand from May 2019 to February

2020 showed among 51 water samples, 41 (80%) contained FLA, of which 28 (55%) were classified as *Acanthamoeba* spp from morphology examination, and among 20 soil samples, 15 (75%) contained FLA, of which 12 (60%) were *Acanthamoeba* spp (Table 1). Cyst morphology of the 40 *Acanthamoeba* spp revealed six (15%) isolates belonging to group I, 20 (50%) to group II and the remaining 14 (35%) to group III.

Molecular identification of *Acanthamoeba* spp

PCR amplification of 18S rDNA (450-bp amplicon) from the 40 isolated *Acanthamoeba* spp described above confirmed the identification of 19 (27%) specimens, 10 from water and nine from soil samples (Table 1). There is no significant difference in the prevalence of waterborne *Acanthamoeba* spp among the five provinces.

Genotyping of *Acanthamoeba* spp

Of the 19 18S rDNA 450-bp *Acanthamoeba*-specific amplicons, 15 were successfully sequenced and classified into genotypes T4 (n = 6), T5 (n = 3), T11 (n = 1), T17 (n = 1), T18 (n = 2), and T20 (n = 2), with 94.3-100 % identity to known genotypes deposited in GenBank (Fig 2) (Coronado-Velázquez *et al*, 2020).

DISCUSSION

A survey of the presence of *Acanthamoeba* spp in water and soil samples were conducted in southern Thailand from May 2019 to February 2020. All three groups of *Acanthamoeba* spp were discovered from observation of cyst morphology. *Acanthamoeba* spp in group I are non-pathogenic except *A. astronyxis, A. byersi* and *A. comandoni* (Qvarnstrom *et al*, 2013; Hajialilo *et al*, 2016), those in group II mostly pathogenic and those group III species often cause infection of the brain, *eg, A. culbertsoni, A. healyi* and *A. lenticulate* (Xuan *et al*, 2017). Thus, the preponderance (85%) of groups II and III *Acanthamoeba* spp collected indicates pathogenic potential of amoebae in southern Thailand.

Acanthamoeba spp can be found in various water and soil habitats across Thailand (Lekkla *et al*, 2005; Thammaratana *et al*, 2016; Putaporntip *et al*, 2021). Presence of *Acanthamoeba* spp in water sources is correlated with the amount of organic matter present (Loret and Greub, 2010) but not with pH or temperature (Putaporntip *et al*, 2021). Our studies found no significant difference in the prevalence of *Acanthamoeba* spp in water samples among the five test provinces in the southern region of the country.

The comparatively lower number of positive *Acanthamoeba* spp identification from FLA using 18S rDNA sequences (27%) compared to morphology observation (56%) reflects the subjective nature of the latter technique. FLA morphology is known to be dependent on culture conditions (Xuan *et al*, 2017). It is accepted

Table 1

Number of free-living amoebae and *Acanthamoeba* spp in water and soil samples collected from southern Thailand (May 2019 - February 2020)

Province	Source	Number of samples	NNA culture, Number (%)		*Acanthamoeba* spp, Number (%) (PCR-based identification)
			FLA	*Acanthamoeba* spp (Morphology-based identification)	
Satun	Water	24	20 (83)	10 (42)	3 (12)
Songkhla	Water	11	10 (91)	10 (91)	3 (27)
Narathiwat	Water	13	8 (61)	7 (54)	3 (23)
	Soil	20	15 (75)	12 (60)	9 (45)
Yala	Water	2	2 (100)	1 (50)	1 (50)
Pattani	Water	1	1 (100)	0 (0)	0 (0)
Total		71	56 (79)	40 (56)	19 (27)

FLA: free-living amoebae; NNA: non-nutrient agar

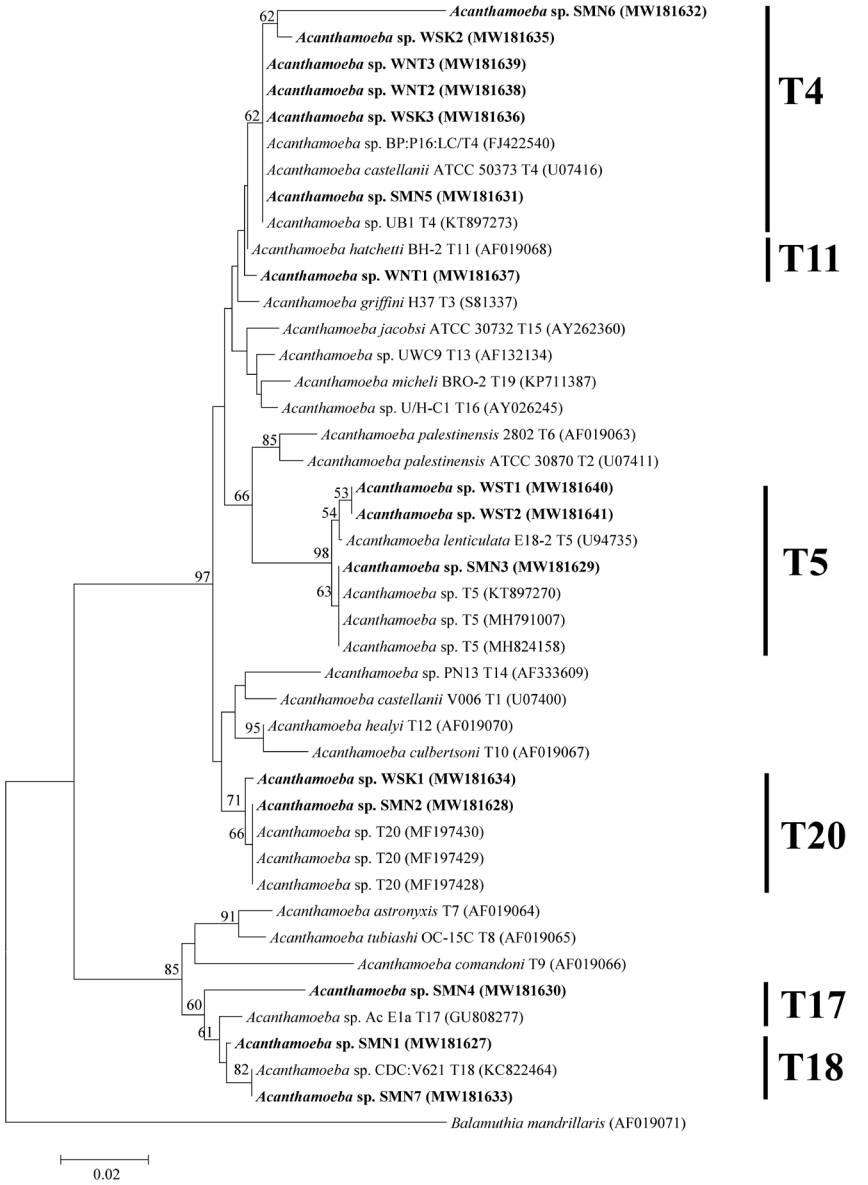

Fig 2 - Phylogenetic grouping of *Acanthamoeba* spp isolated from water and soil samples in southern Thailand (May 2019 - February 2020)

Phylogenetic tree was constructed from 18S rDNA sequences (450 bp) of genus Acanthamoeba using a neighbor-joining distance method with Balamuthia mandrillaris as an outgroup. Bootstrap value (1,000 replications) is shown at node. *Acanthamoeba* sp from the study is indicate in bold and T value indicates genotype (Coronado-Velázquez *et al*, 2020). GenBank accession number is shown in parenthesis. SMN indicates specimens obtained from soil samples. WNT, WSK, WST indicate specimens obtained from water samples. Scale bar represents 0.02 substitution per nucleotide position.

that, in the diagnosis of AK clinical cases, sensitivity of the typical culture method is poor compared to that of the PCR-based method (Hoffman *et al*, 2021) and, thus, a molecular method is crucial for accurate species identification as well as genotyping of *Acanthamoeba*.

Following molecular detection and sequencing, six *Acanthamoeba* genotypes (T4, T5, T11, T17, T18, and T20) were identified. Genotype T4 is thought to be the genotype mainly associated with AK in both contact lens wearers and non-wearers (Taher *et al*, 2018; de Lacerda and Lira, 2021). The predominance of T4 isolates from soil and water samples in our study (40%) is similar to that in environmental samples reported from China (Lass *et al*, 2017; Xuan *et al*, 2017). T5 genotype was reported to be the etiology of the death of a 39-year-old heart transplant patient (Barete *et al*, 2007) and has been associated with corneal infection (Siddiqui and Khan, 2012). The sequence of a T11 isolate (sequence code WNT1) from a water sample is identical to *A. hatchetti* BH-2, which has been associated with cases of AK worldwide (Niyyati *et al*, 2009; Lorenzo-Morales *et al*, 2011; Lorenzo-Morales *et al*, 2015). A T17 isolate (sequence code SMN4) from a soil sample from Narathiwat Province was previously reported in water sources in other regions of the country (Nuprasert *et al*, 2010) and in drinking water from Khyber Pakhtunkhwa, Pakistan (Tanveer *et al*, 2013), but is believed to be non-pathogenic (Siddiqui and Khan, 2012). Soil from Narathiwat Province also harbored two T18 isolates (sequence codes SMN1 and SMN7), a genotype reported to cause GAE (Matsui *et al*, 2018). T20, another genotype of highly pathogenic, GAE-causing strain (Fuerst *et al*, 2015), was isolated from a water (sequence code WSK1) and a soil (sequence code SMN2) sample.

Although previous studies have reported the presence of *Acanthamoeba* spp in natural water sources in central, northern and southern regions of the country (Lekkla *et al*, 2005; Nuprasert *et al*, 2010), the present findings serve as additional evidence of the presence of pathogenic *Acanthamoeba* spp in environmental sources directly connected to human activities. The high level of amoeba contamination of both water and soil with potentially pathogenic genotypes in the regions surveyed should be considered to pose an infection risk, particularly to immunocompromised individuals and contact lens wearers. Public awareness of possible *Acanthamoeba* infection and appropriate precautionary measures should be undertaken. The possibility of amoeba cysts spreading through water and soil and in air through aerosols should also be addressed.

In conclusion, this is the first demonstration of the presence of *Acanthamoeba* genotypes T17, T18 and T20 in water and soil in southern

Thailand, and the high frequency in this region of the country of *Acanthamoeba* spp in morphology groups II and III and genotypes T4, T5, T11, and T18, associated with *Acanthamoeba* keratitis and granulomatous amoebic encephalitis, should be noted.

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CONFLICTS OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

REFERENCES

Barete S, Combes A, de Jonckheere JF, *et al*. Fatal disseminated *Acanthamoeba lenticulate* infection in a heart transplant patient. *Emerg Infect Dis* 2007; 13: 736-8.

Bunsuwansakul C, Mahboob T, Hounkong K, *et al*. *Acanthamoeba* in Southeast Asia - overview and challenges. *Korean J Parasittol* 2019; 57: 341-57.

Carnt NA, Subedi D, Connor S, Kilvington S. The relationship between environmental sources and the susceptibility of *Acanthamoeba* keratitis in the United Kingdom. *PLoS One* 2020; 15: e0229681.

Chuckpaiwong V, Vathesatokkit C, Lekhanont K, Vongthongsri A. *Acanthamoeba* keratitis: character and outcome of treatment in tropical area. *Thai J Opthalmol* 2010; 24: 26-36.

Coronado-Velázquez D, Silva-Olivares A, Castro-Munozledo F, *et al*. *Acanthamoeba mauritaniensis* genotype T4D: an environmental isolate displays pathogenic behavior. *Parasitol Int* 2020; 74: 102002.

de Lacerda AG, Lira M. *Acanthamoeba* keratitis: a review of biology, pathophysiology and epidemiology. *Ophthalmic Physiol Opt* 2021; 41: 116-35.

Fabres LF, Rosa dos Santos SP, Benitez LB, Rott MB. Isolation and identification of *Acanthamoeba* spp. from thermal swimming pools and spas in Southern Brazil. *Acta Parasitol* 2016; 61: 221-7.

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: 69-84.

Hajialilo E, Behnia M, Tarighi F, Niyyati M, Rezaeian M. Isolation and genotyping of *Acanthamoeba* strains (T4, T9, and T11) from amoebic keratitis patients in Iran. *Parasitol Res* 2016; 115: 3147-57.

Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucl Acids Symp Ser* 1999; 41: 95-8.

Hoffman JJ, Dart JKG, De SK, Carnt N, Cleary G, Hau S. Comparison of culture, confocal microscopy and PCR in routine hospital use for microbial keratitis diagnosis. *Eye* 2021; doi: 10.1038/s41433-021-01812-7. Online ahead of print.

Khan NA. *Acanthamoeba*: biology and

increasing importance in human health. *FEMS Microbiol Rev* 2006; 30: 564-95.

Lackner P, Beer R, Broessner G, *et al*. Acute granulomatous *Acanthamoeba* encephalitis in an immunocompetent patient. *Neurocrit Care* 2010; 12: 91-4.

Lass A, Guerrero M, Li X, Karanis G, Ma L, Karanis P. Detection of *Acanthamoeba* spp. in water samples collected from natural water reservoirs, sewages, and pharmaceutical factory drain using LAMP and PCT in China. *Sci Total Environ* 2017; 584-585: 489-94.

Lekkla A, Sutthikornchai C, Bovornkitti S, Sukthana Y. Free-living ameba contamination in natural hot springs in Thailand. *Southeast Asian J Trop Med Public Health* 2005; 36 (Suppl 4): 5-9.

Li W, Wang Z, Qu J, Zhang Y, Sun X. *Acanthamoeba* keratitis related to contact lens use in a tertiary hospital in China. *BMC Ophthalmol* 2019; 19): 202.

Lorenzo-Morales J, Khan NA, Walochnik J. An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. Parasite 2015; 22: 10.

Lorenzo-Morales J, Morcillo-Laiz R, Martin-Navarro CM, *et al*. *Acanthamoeba* keratitis due to genotype T11 in a rigid gas permeable contact lens wearer in Spain. *Cont lens Anterior Eye* 2011; 34: 83-6.

Loret JF, Greub G. Free-living amoebae: biological by-passes in water treatment. *Int J Hyg Environ Health* 2010; 213: 167-75.

Matsui T, Maeda T, Kusakabe S, *et al*. A case report of granulomatous amoebic encephalitis by group 1 *Acanthamoeba* genotype T18 diagnosed by the combination of morphological examination and genetic analysis. *Diagn Pathol* 2018; 13: 27.

Mohaghegh MA, Azimi Resketi M, Mohammadimanesh R, *et al*. Soil contamination with free-living amoeba in north of Iran. *Int J Infect* 2016; 3: e37923.

Niyyati M, Lorenzo-Morales J, Rezaie S, *et al*. Genotyping of *Acanthamoeba* isolates from clinical and environmental specimens in Iran. *Exp Parasitol* 2009; 121: 242-5.

Nuprasert W, Putaporntip C, Pariyakanok L, Jongwutiwes S. Identification of a novel T17 genotype of *Acanthamoeba* from environmental isolates and T10 genotype causing keratitis in Thailand. *J Clin Microbiol* 2010; 48: 4636-40.

Pussard M, Pons R, Mas-Pons R. Morphologies of cystic wall and taxonomy of genus *Acanthamoeba* (Protozoa, Amoebida). *Protistologica* 1977; 13: 557-98. [in French]

Putaporntip C, Kuamsab N, Nuprasert W, *et al*. Analysis of *Acanthamoeba* genotypes from public freshwater sources in Thailand reveals a new genotype, T23 *Acanthamoeba bangkokensis* sp. nov. *Sci Rep* 2021; 11: 17290.

Qvarnstrom Y, Nerad TA, Visvesvara GS. Characterization of a new pathogenic *Acanthamoeba* species, *A. byersi* n. sp., isolation from a human with fetal amoebic encephalitis. *J Eukaryot Microbiol* 2013; 60: 626-33.

Schroeder JM, Booton GC, Hay J, *et al*. Use of

subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of acanthamoebae from humans with keratitis and from sewage sludge. *J Clin Microbiol* 2001; 39: 1903-11.

Schuster FL. Cultivation of pathogenic and opportunistic free-living amebas. *Clin Microbiol Rev* 2002; 15: 342-54.

Siddiqui R, Khan NA. Biology and pathogenesis of *Acanthamoeba*. *Parasit Vectors* 2012; 5: 6.

Taher EE, Méabed EMH, Abdallah I, Abdel Wahed WY. *Acanthamoeba* keratitis in noncompliant soft contact lenses users: genotyping and risk factors, a study from Cairo, Egypt. *J Infect Public Health* 2018; 11: 377-83.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 2013; 30: 2725-9.

Tananuvat N, Techajongjintana N, Somboon P, Wannasan A. The first *Acanthamoeba* keratitis case of non-contact lens wearer with HIV infection in Thailand. *Korean J Parasitol* 2019; 57: 505-11.

Tanveer T, Hameed A, Muazzam AG, Jung SY, Gul A, Matin A. Isolation and molecular characterization of potentially pathogenic *Acanthamoeba* genotypes from diverse water resources including household drinking water from Khyber Pakhtunkhwa, Pakistan. *Parasitol Res* 2013; 112: 2925-32.

Thammaratana T, Laummaunwai P, Boonmars T. Isolation and identification of *Acanthamoeba* species from natural water sources in the northeastern part of Thailand. *Parasitol Res* 2016; 115: 1705-9.

Wanachiwanawin D, Booranapong W, Kosrirukvongs, P. Clinical feature of *Acanthamoeba* keratitis in contact lens wearers and non-wearers. *Southeast Asian J Trop Med Public Health* 2012; 43: 549-56.

Wannasan A, Uparanukraw P, Songsangchun A, Morakote N. Potentially pathogenic free-living amoebae in some flood-affected areas during 2011 Chiang Mai flood. *Rev Inst Med Trop Sao Paulo* 2013; 55: 411-6.

Winck MA, Caumo K, Rott MB. Prevalence of *Acanthamoeba* from tap water in Rio Grande do Sul, Brazil. *Curr Microbiol* 2011; 63: 464-9.

Xuan Y, Shen Y, Ge Y, Yan G, Zheng S. Isolation and identification of *Acanthamoeba* strains from soil and tap water in Yanji, China. *Environ Health Prev Med* 2017; 22: 58.