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Acanthamoeba keratitis: Molecular typing of *Acanthamoeba* species directly from ocular tissue

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ABSTRACT

This report explores the molecular profiling of *Acanthamoeba* spp. from individuals in the UK suffering from a debilitating, sight-threatening disease of the cornea known as *Acanthamoeba* keratitis (AK). Seventy ocular samples from individuals undergoing investigations for AK were sent to the Scottish Microbiology Reference Laboratories (SMiRL), Glasgow during 2017–2019, and subjected to DNA extraction followed by in-depth molecular typing using a nested PCR/bi-directional sequencing approach. Of the 70 samples tested, 40 were PCR-positive. Of these, 32 were successfully sequenced and assigned to two of 23 existing genotypes termed T1 to T23. Molecular profiling of the 32 samples highlighted two genotypes, namely T3 ($n = 3$) and T4 ($n = 29$). For those 29 samples identified as the T4 genotype, a sub-genotype (T4A-T4H) was recorded: T4A ($n = 18$); T4B ($n = 5$); T4C ($n = 1$); T4E ($n = 4$); and T4F ($n = 1$). This study highlights that the T4 genotype and T4A subtype are the predominant molecular variants to cause ocular disease in the UK. Gaining in-depth information on the molecular profiling of *Acanthamoeba* spp. is essential to increase our understanding of the source(s) of infection, transmission pathways, and potential associations with clinical outcomes for this rare, yet potentially debilitating ocular disease.

1. Introduction

Acanthamoeba spp. are ubiquitous, free-living microorganisms that can cause a serious eye infection in humans termed *Acanthamoeba* keratitis (AK). First reported in the UK in 1974 (Jones et al., 1975), common symptoms include ocular pain, irritation, blurred vision, sensitivity to light, and excessive tearing. Symptoms can be prolonged and, depending on the severity of the infection, can lead to partial or total sight loss. *Acanthamoeba* keratitis is most commonly associated with contact lens use in approximately 80–85% of UK cases (Carnt and Stapleton, 2016) and has been reported elsewhere in non-contact lens wearers (Garg et al., 2017). Risk factors associated with AK include poor hygiene during handling and cleaning of contact lenses, rinsing lenses with contaminated tap water, bathing or swimming whilst wearing contact lenses, and damage to the eye (Roshni Prithiviraj et al., 2020).

An AK diagnosis is based on a combination of clinical presentation and laboratory identification. Due to the rarity of the disease, managing AK can be extremely challenging as it is not always considered in the first-line differential diagnosis. Symptoms may be mistaken for those caused by other, more common ocular pathogens including herpes simplex virus (HSV), or individuals may present with co-infections. Although molecular technologies are gradually being implemented across the UK within specialist centres to assist with an AK diagnosis, many National Health Service (NHS) laboratories still attempt to culture *Acanthamoeba* spp. from corneal tissue. This involves the use of non-nutrient agar plates seeded with *Escherichia coli*, where feeding tracks are observed if *Acanthamoeba* spp. are present. This technique has its limitations as a positive result can be difficult to interpret and may require several weeks of prolonged incubation which can have a detrimental clinical impact. Other testing methods include direct microscopy to detect cysts and trophozoites which can be performed using a variety of stains to enhance their appearance (Le Govic et al., 2018). Confocal microscopy is also a useful tool to aid diagnosis but requires advanced training and expertise.

In recent years, advances in laboratory techniques have improved the diagnosis of AK which is crucial, especially as reports of this disease within the UK have increased over the past ten years (Carnt et al., 2020). These include the implementation of molecular tests such as real-time

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PCR assays (Rivière et al., 2006; Alexander et al., 2015) and conventional PCR assays (Schroeder et al., 2001; Booton et al., 2002; Di Cave et al., 2009). These rapid and sensitive molecular assays provide in-depth data by amplifying the 18S ribosomal ribonucleic acid (rRNA) small subunit region known as DF3 (diagnostic fragment 3, approximately 240 nucleotides long). This is widely used for the genotyping of *Acanthamoeba* spp. To date, there are 31 *Acanthamoeba* spp. and at least 23 existing genotypes termed T1 to T23. Those identified as the T4 genotype can be further subdivided into T4A-T4H (Corsaro, 2020).

Most published molecular assays that permit *Acanthamoeba* spp. molecular profiling, amplify DNA from cultures where there is an abundance of DNA copies. However, culturing of this organism is not particularly sensitive (Lorenzo-Morales et al., 2015) and can introduce bias as certain variants may prefer growing on selected agar, and some variants may not grow at all. The most common genotype identified from cultured isolates is T4 which has been shown to be related to keratitis infections. Rather than using cultured isolates, this study explores the molecular variants of *Acanthamoeba* spp. isolated directly from ocular material from laboratory-confirmed AK cases using a nested PCR/sequence-based assay which detects the following variants: T3, T4, T7, T8, T10, T11, T12 and T15. We describe a snapshot of molecular variants that cause AK in the UK and raise awareness of this debilitating disease. The outputs form the foundations to improve our understanding of associations between variants and clinical outcomes.

2. Materials and methods

2.1. Clinical specimens

A total of 70 ocular samples from 70 human cases under investigation for AK during 2017–2019 were referred to the SMiRL, Glasgow from Microbiology and Ophthalmology departments from nine National Health Service (NHS) Heath Boards within Scotland and one NHS Foundation Trust in England as part of routine testing. Forty samples were submitted from Scottish residents and 30 from England, which comprised 65 corneal tissues, 1 contact lens, 2 contact lens fluids and 2 corneal swabs. No patient identifiers were disclosed to protect patient identity and confidentiality, and samples formed part of the SMiRL, Glasgow national reference collection.

2.2. DNA extraction

DNA from each sample was subjected to silica-based semi-automatic DNA extraction (easyMAG, BioMérieux, France), following the manufacturer's Generic A protocol. A sample of 200 µl was extracted and the DNA eluted in a final volume of 100 µl.

2.3. Typing assay

DNA lysates from 70 cases under investigation for AK were considered for genotyping using a nested PCR/sequencing molecular assay based on modifications of a previously published assay which amplified the 18S ribosomal RNA (rRNA) subunit (Rns) gene (Booton et al., 2002). To optimise the amplification of DNA directly from corneal samples, the nested PCR assay incorporated newly designed outer PCR primers (Round 1 primers, labelled AGCF and AGCR) whilst the inner PCR primers (Round 2 primers) were those previously described (Booton et al., 2002) (Table 1). The Round 1 PCR protocol consisted of 2× AccuStart™ II PCR ToughMix® (VWR, Leicester, UK), 3.125 µM primers (final concentration of 0.1875 µM), molecular grade water, and 5 µl of DNA in a 25 µl reaction volume. The Round 2 PCR amplification used primers JDP1 and JDP2 (Table 1), and the PCR protocol consisted of 5 µl of PCR1 product, 2× AccuStart™ II PCR ToughMix® (VWR, Leicester, UK), 6.25 µM primers (final concentration 0.375 µM) and molecular grade water in a total reaction volume of 50 µl. Appropriate negative and positive controls were included. Cycling conditions consisted of 96 °C for 2 min, followed by 40 cycles of 96 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and 72 °C for 7 min. Amplification was performed in a GeneAmp System 9700 thermocycler (Applied Biosystems, Warrington, UK). Five microlitres of the second-round PCR amplicon (~450 bp) was resolved by agarose gel electrophoresis and visualized by UV transillumination after electrophoresis at 80 V for 1 h in 1.4% agarose gels stained with ethidium bromide (0.5 µg/ml). Gels were photographed using the Gel Doc 2000 system equipped with QuantityOne software (Bio-Rad, Watford, UK) for gel documentation.

Table 1
Sequences of *Acanthamoeba* spp. primers for the nested PCR/sequence-based assay.

Assay	Oligonucleotide	Sequence (5′-3′)	Reference
Nested PCR assay (Round 1)	AGCF AGCR	CAACACTCCGGTGGGTGGTCT CCCGGAACCCAAAGACTTATG	Present study
Nested PCR assay (Round 2)	JDP1 JDP2	GGCCCAGATCGTTTACCGTGAA TCTCACAAGCTGCTAGGGGAGTCA	Booton et al. (2002)

2.4. Sequencing

Positive PCR amplicons were purified with polyethylene glycol and 2.5 M sodium chloride (20% PEG-2.5 M NaCl) to remove residue dNTPs and primers. Sanger sequencing was performed for both forward and reverse primers individually using Big Dye Terminator version 3.1 chemistry. Cyclic PCR reactions were performed in 10 µl volume with the following thermal cycling conditions: an initial denaturation step at 96 °C for 2 min followed by 28 cycles of denaturation at 96 °C for 10 s, annealing at 60 °C for 10 s and an extension at 60 °C for 4 min. The PCR products were purified with cold 100% ethanol and 3M sodium acetate (EtOH/salt) mix, and the sequences were read in an automated capillary DNA sequencer model 3500xL genetic analyser (Applied Biosystems, Warrington, UK).

2.5. Sequence analysis

Bidirectional Sanger sequences from each PCR product were assembled into contigs with the EMBOSS Needle Pairwise Sequence Alignment tool from EBI (https://www.ebi.ac.uk/Tools/psa/emboss_needle/). BioEdit (Hall, 1999) was used to manually check and correct the contig consensus sequences based on the Sanger chromatograms. Each consensus sequence was searched against NCBI GenBank using BLAST and provisionally assigned to a genotype based on the closest hits.

Phylogenetic analysis was then used to confirm the genotype assignment and further resolve sub-genotypes within T4. Sequences were aligned using MUSCLE and trimmed to the stem 29-1 diagnostic region of the DF3 section of the 18S rRNA gene. These were then aligned to the *Acanthamoeba* stem 29-1 allele database (sequences for T4, T3, and T11), available at https://u.osu.edu/acanthamoeba/. This alignment, consisting of 225 sequences, was used to compute a maximum likelihood phylogenetic tree in RAxML (Stamatakis, 2015), using the Kimura 2-parameter model and 100 bootstrap replicates. The resulting consensus tree was visualized and annotated with iTOL v.6.8 (Letunic and Bork, 2021).

3. Results and discussion

Of the 70 samples tested, 40 generated an amplicon of ~450 bp using the modified nested PCR/sequence-based assay (data not shown). High-

quality sequences were obtained for 32 of these samples (corneal tissue, $n = 29$; contact lens, $n = 1$; and contact lens fluid, $n = 2$). Thirty-two were successfully assigned a genotype (Table 2). Eight of the samples that generated an amplicon, did not produce sequences of sufficient quality. Three samples (contact lens fluid, $n = 1$; corneal tissue, $n = 2$) were identified as the T3 genotype (Table 2, Fig. 1). The remaining 29 samples (corneal tissue, $n = 27$; contact lens, $n = 1$; and contact lens fluid, $n = 1$) were found to have the most common T4 variant; these were categorised into the following 5 specific subtypes: T4A, B, C, E and F (Table 2, Fig. 1). Three clusters of T4A were obtained: (T4(1), $n = 13$); T4(2), $n = 1$; and T4(3), $n = 4$) (Fig. 1). Genotype examples were made available on GenBank under accession numbers: T3 (MT944213); T4A (MT944209; MT944202; MT944211; MT944203; MT944204; MT944205; MT944206; MT944207; MT944210); T4B (OR257994); T4C (MT944212); T4E (OR257995); and T4F (OR257996).

We report on the molecular profiles of *Acanthamoeba* spp. responsible for causing ocular disease from a UK cohort of individuals using DNA amplified directly from clinical material. A recent study in the UK described the presence of *Acanthamoeba* spp. DNA in AK patients; however, no molecular typing was performed (Carnt et al., 2020). To date, there has been very limited information on *Acanthamoeba* spp. variants that cause AK in the UK, with one study examining six cases where DNA was extracted from cultured isolates, rather than directly from clinical material (Maghsood, et al., 2005). Culturing requires samples to grow successfully, and previous reports have shown that the ability to culture *Acanthamoeba* spp. is highly variable with success rates of 30–50% (Goh et al., 2018). In addition, certain variants may favour culture conditions better than others resulting in a degree of bias as dominant types prevail. Similar to Yera et al. (2021) and Holmgaard et al. (2021), in our study we identified genotypes from extremely small starting quantities of clinical material taken directly from the clinical sample. Advantages of this method include removing the need to culture therefore excluding any bias that may arise from this process, having a higher amplification success rate, and achieving results rapidly within 24–48 h to benefit patient management.

Previous studies have shown that well-established primers have an amplification rate of 14–68% (Booton et al., 2002; Di Cave et al., 2009). Our nested PCR/sequence-based molecular assay amplified DNA from 40 of the 70 samples tested. It is likely that the remaining 30 samples which were PCR-negative, were from cases where symptoms were caused by a pathogen(s) other than *Acanthamoeba* spp. as the clinical presentation can be similar for infections including HSV (Singh and Batta, 2018). Of course, a limitation of the assay is that it does not detect every genotype (T2, T5 and T6, data not shown); therefore, there is the possibility that a subset of the PCR-negative samples contained *Acanthamoeba* spp. genotypes not detected by the assay. However, as the majority of reports highlight the dominant AK-causing genotype to be T4, it is most likely these PCR-negative samples were truly negative for *Acanthamoeba* spp. DNA. Of the 40 PCR-positive samples, eight were not assigned a genotype due to poor quality sequence despite amplicons being produced. This may be explained by the presence of other pathogen DNA in the sample as a result of a mixed infection or could be due to inhibitor(s) that impact downstream molecular events. One study reported that topical agents and corneal stains used during investigations for AK were PCR inhibitory (Goldschmidt et al., 2006). In addition, the most common topical eye drops to treat AK contains polyhexamethylene biguanide which can also inhibit molecular reactions (Thompson et al., 2008). It is not known if the two cases that we were unable to sequence had previously been treated for AK prior to a laboratory-confirmed diagnosis.

Table 2
Acanthamoeba spp. genotypes identified and their geographical distribution.

Genotype	Scotland (*n*)	England (*n*)	Total
T3	2	1	3
T4A	12	6	18
T4B	4	1	5
T4C	0	1	1
T4E	1	3	4
T4F	0	1	1
Total	19	13	32

Abbreviation: n, number of cases.

Only one other UK study from over 15 years ago reported typing data where five cases belonged to the T4 genotype and one case belonged to the T3 genotype in AK patients (Maghsood et al., 2005). This is supportive of our findings where 29 cases were found to be this type. The T4 genotype is closely related to the T3 (*A. griffini*) genotype which is also associated with AK (Maciver et al., 2013), and has been shown to have resistance to multipurpose contact lens cleaning solution (Zhang et al., 2023). Previously, Roshni Prithiviraj et al. (2020) identified four T4 subtypes (T4A, T4B, T4D and T4E) in people with AK in India. In comparison, in our study, five subtypes were detected (T4A, T4B, T4C, T4E and T4F), with T4A being the most frequently found. *Acanthamoeba* spp. that cause AK are known to be present in a variety of water sources, i.e. drinking (tap and bottled) and recreational (swimming pools, spas and beaches), well water and groundwater (de Lacerda and Lira, 2021; Masangkay et al., 2022). Reports performing genotyping on water samples have indicated that genotypes are present which are known to cause human disease including the dominant T4 genotype (Putapporntip et al., 2021; Padua et al., 2023). In addition to T4, we report the T3 genotype from individuals presenting with AK, which is similar to previous reports from three continents supporting its association with this disease (Diehl et al., 2021).

In terms of potential risk factors, of interest, only 12 out of the 40 PCR-positive cases in this study were stated to wear contact lenses based on the limited information recorded on each laboratory request form. This is lower than expected as a previous report cited contact lens use occurred in around 80% of AK cases in the UK (Carnt and Stapleton, 2016). Of course, it is possible that this information was simply not recorded on the laboratory request forms, which is not uncommon. Or it may represent a much higher infection rate than previously thought in non-contact lens wearers. Roshni Prithiviraj et al. (2020) described a recent study of 30 individuals and highlighted that risk factors other than contact lens use were present in AK cases. These included having a foreign body in the eye, bathing in pond water, injuries caused by sticks, leaves or stones, and insect bites. Within the UK, domestic tap water has been identified as a possible risk factor following an outbreak (Kilvington et al., 2004). At present, information on laboratory request forms is often limited which restricts the quantity and quality of data captured for each case. There is no enhanced surveillance for AK in the UK to support national robust data collection for every case. Having a pathway to capture information on associated risks (e.g. water exposure, hand hygiene, contact lens practices, soil contact, injury, occupation, and travel) would improve our understanding of this disease, to permit the exploration of clinical associations with specific molecular variants.

Of note, seven patients within our cohort were non-responsive to the standard treatment regime. Treatment for AK needs to be administered promptly to minimize disease progression and is often given over lengthy periods. Treatment failure can arise for a variety of reasons including the premature ceasing of antimicrobials, non-compliance with treatment regimes, or drug resistance. In the small number of drug-resistant cases, the lack of response increases the risk of the disease progressing, which may result in corneal transplants or, at worse, complete loss of sight in the infected eye. This has been highlighted recently by Connelly et al. (2019) who described a non-responsive case that resulted in the affected individual receiving two corneal grafts. It is unclear if the disease severity is associated with specific species and/or genotypes. A recent study suggested disease severity was not linked to specific genetic variants (Roshni Prithiviraj et al., 2020). However, Arnalich-Montiel et al. (2014) indicated that subtypes T3 and T11 are more virulent than the T4 genotype. This study also indicated that non-T4 patients responded less well to treatment, and surgical

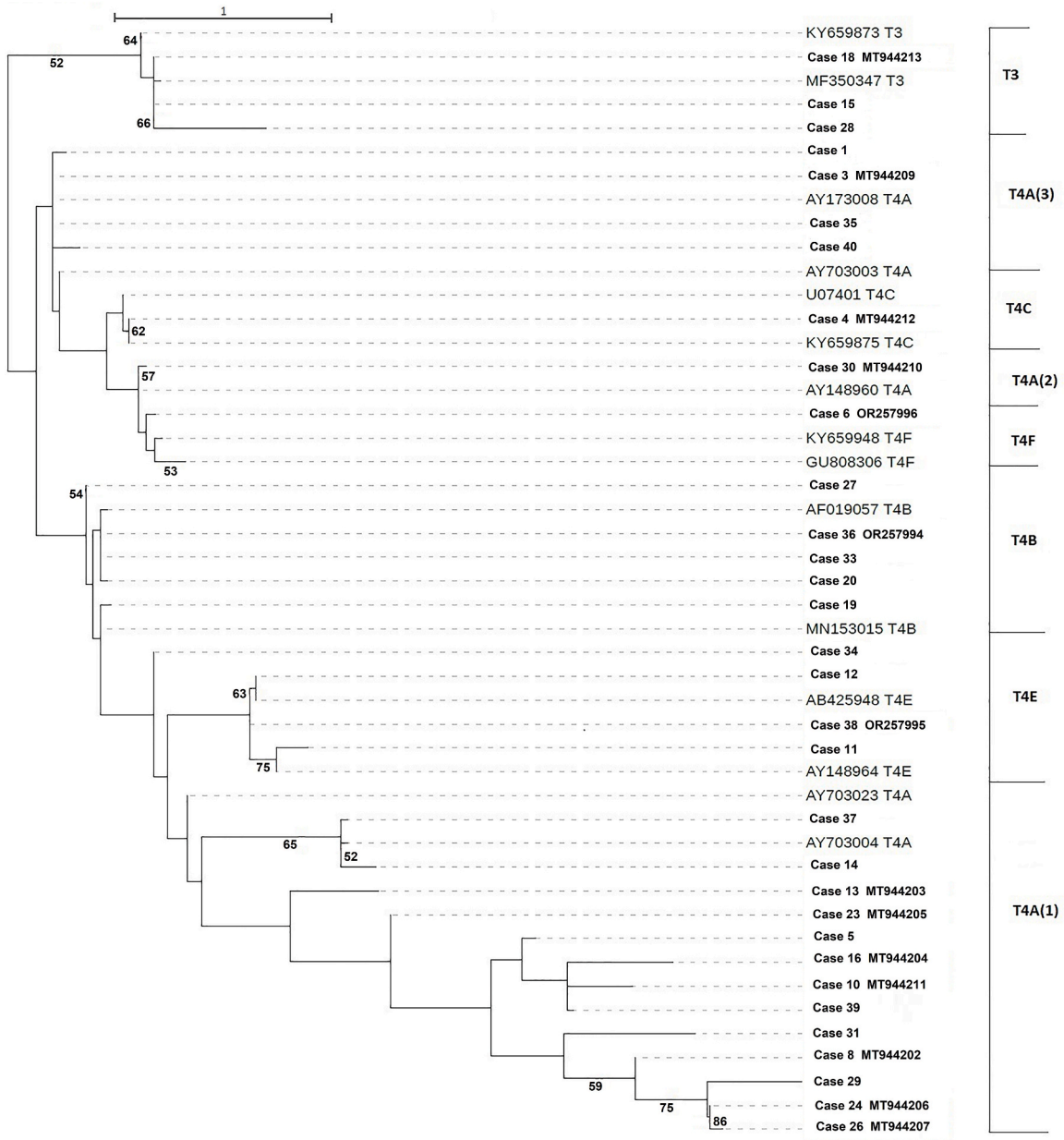


Fig. 1. Maximum likelihood phylogenetic tree of *Acanthamoeba* spp. based on 18S rRNA gene (stem 29-1 DF3 region). Sequences from this study are highlighted in bold. Reference sequences from known genotypes were obtained from the *Acanthamoeba* 18S rRNA DNA database (https://u.osu.edu/acanthamoeba/). The tree was computed with RAxML using the Kimura 2-parameter model and 100 bootstrap replicates. Bootstrap values are shown for nodes with > 50% support. The tree was computed with an alignment of 225 sequences (including those from the present study and reference sequences), but for clarity, only the reference sequences closest to the sequences from this study are displayed. The scale-bar indicates the number of nucleotide substitutions per site.

intervention was required. Further work is required on much larger cohorts to explore the association between the molecular variants and disease severity. Out of the seven individuals in our study who failed to respond to anti-microbial treatment, four patients were infected with T4A and the other three with T4B. It is possible that these represent novel, virulent *Acanthamoeba* spp., as has recently been described by Putaporntip et al. (2021). Further work to explore possible genetic markers to assist patient management would be beneficial as these cases represent those that are challenging and are likely to result in a poorer outcome.

4. Conclusions

This report raises awareness of AK which is both difficult to diagnose and can be challenging to manage. We report on the findings of a modified typing assay which can be utilised to determine the molecular variants of *Acanthamoeba* spp. directly from a clinical sample. This has allowed us to determine that the two predominant variants in the UK are currently T4 and T3, and five T4 subtypes exist. To improve our understanding of *Acanthamoeba* spp. in clinical disease, it is important to address various challenges. Due to the relative rarity of the disease, there is a limited number of studies across the globe, often describing very small cohorts of individuals. To gain a better understanding of the global diversity of *Acanthamoeba* spp., there is a need for a managed sequencing database to capture accurate and concise molecular information to allow robust comparisons to be made on a much wider scale. Further improvements include the introduction of an enhanced surveillance system, as has been performed for other parasites in the UK including *Giardia duodenalis*, *Schistosoma* spp., and *Plasmodium* spp., to ensure high quality, in-depth information is captured for every case.

This will aid clinicians and scientists to have a much greater understanding of possible sources and transmission routes to minimize further cases and improve patient management. Furthermore, going forward, there is a need for studies examining potential sources within the UK, and elsewhere, to improve source tracing which is essential to prevent infection and spread of this debilitating disease.

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Ethical approval

Not applicable. This study constituted continuous quality improvement as part of the remit of the Scottish Microbiology Reference Laboratories, Glasgow. No additional samples were requested for this study, no patient identifiers were stated, and none of the information was used to inform clinical management.

CRediT authorship contribution statement

Lisa Connelly: Methodology, Validation, Investigation, Data Curation, Writing - original draft, Writing - review & editing. **Deepa Anijeet:** Resources, Writing - review & editing. **Derek Tole:** Resources, Writing - review & editing. **Claire L. Alexander:** Conceptualization, Writing - review & editing, Supervision, Project administration.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The sequences generated in the present study were submitted to the GenBank database under the accession numbers MT944202-MT944207, MT944209-MT944213 and OR257994-OR257996.

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References

Alexander, C.L., Coyne, M., Jones, B., Anijeet, D., 2015. *Acanthamoeba* keratitis: improving the Scottish diagnostic service for the rapid molecular detection of *Acanthamoeba* species. J. Med. Microbiol. 64, 682–687.

Arnalich-Montiel, F., Lumbreras-Fernández, B., Martín-Navarro, C.M., Valladares, B., Lopez-Velez, R., Morcillo-Laiz, R., et al., 2014. Influence of *Acanthamoeba* genotype on clinical course and outcomes for patients with *Acanthamoeba* keratitis in Spain. J. Clin. Microbiol. 52, 1213–1216.

Booton, G.C., Kelly, D.J., Chu, Y.W., Seal, D.V., Houang, E., Lam, D.S.C., et al., 2002. 18S ribosomal DNA typing and tracking of *Acanthamoeba* species isolates from corneal scrape specimens, contact lenses, lens cases, and home water supplies of *Acanthamoeba* keratitis patients in Hong Kong. J. Clin. Microbiol. 40, 1621–1625.

Carnt, N., Stapleton, F., 2016. Strategies for the prevention of contact lens-related *Acanthamoeba* keratitis: a review. Ophthalmic Physiol. Opt. 36, 77–92.

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

Connelly, L., Anijeet, D., Alexander, C.L., 2019. A descriptive case of persistent *Acanthamoeba* keratitis: raising awareness of this complex ocular disease. Access Microbiol 2, acmi000084.

Corsaro, D., 2020. Update on *Acanthamoeba* phylogeny. Parasitol. Res. 119, 3327–3338. https://doi.org/10.1007/s00436-020-06843-9.

de Lacerda, A.G., Lira, M., 2021. *Acanthamoeba* keratitis: a review of biology, pathophysiology and epidemiology. Ophthalmic Physiol. Opt. 41, 116–135.

Di Cave, D., Monno, R., Bottalico, P., Guerriero, S., D'Amelio, S., D'Orazi, C., et al., 2009. *Acanthamoeba* T4 and T15 genotypes associated with keratitis infections in Italy. Eur. J. Clin. Microbiol. Infect. Dis. 28, 607–612.

Diehl, M.L.N., Paes, J., Rott, M.B., 2021. Genotype distribution of *Acanthamoeba* in keratitis: a systematic review. Parasitol. Res. 120, 3051–3063.

Garg, P., Kalra, P., Joseph, J., 2017. Non-contact lens related *Acanthamoeba* keratitis. Indian J. Ophthalmol. 65, 1079–1086.

Goh, J.W.Y., Harrison, R., Hau, S., Alexander, C.L., Tole, D.M., Avadhanam, V.S., 2018. Comparison of *in vivo* confocal microscopy, PCR and culture of corneal scrapes in the diagnosis of *Acanthamoeba* keratitis. Cornea 37, 480–485.

Goldschmidt, P., Rostane, H., Saint-Jean, C., Batellier, L., Alouch, C., Zito, E., et al., 2006. Effects of topical anaesthetics and fluorescein on the real-time PCR used for the diagnosis of herpesviruses and *Acanthamoeba* keratitis. Br. J. Ophthalmol. 90, 1354–1356.

Hall, T.A., 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Holmgaard, D.B., Barnadas, C., Mirbarati, S.H., Andersen, L.O.B., Nielsen, H.V., Stensvold, C.R., et al., 2021. Detection and identification of *Acanthamoeba* and other nonviral causes of infectious keratitis in corneal scrapings by real-time PCR and next-generation sequencing-based 16S-18S gene analysis. J. Clin. Microbiol. 59, e02224-20.

Jones, D.B., Visvesvara, G.S., Robinson, N.M., 1975. *Acanthamoeba polyphaga* keratitis and *Acanthamoeba* uveitis associated with fatal meningoencephalitis. Trans. Ophthalmol. Soc. U. K. 95, 221–232.

Kilvington, S., Gray, T., Dart, J., Morlet, N., Beeching, J.R., Frazer, D.G., et al., 2004. *Acanthamoeba* keratitis: the role of domestic tap water contamination in the United Kingdom. Invest. Ophthalmol. Vis. Sci. 45, 165–169.

Le Govic, Y., Gonçalves, A., de Gentile, L., Pihet, M., 2018. Common staining techniques for highlighting *Acanthamoeba* cysts. Clin. Microbiol. Infect. 24, 970–971.

Letunic, I., Bork, P., 2021. Interactive Tree of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 49 (W1), W293–W296. https://doi.org/10.1093/nar/gkab301.

Lorenzo-Morales, J., Khan, N.A., Walochnik, J., 2015. An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. Parasite 22, 10–29.

Maciver, S.K., Asif, M., Simmen, M.W., Lorenzo-Morales, J., 2013. A systematic analysis of *Acanthamoeba* genotype frequency correlated with source and pathogenicity: T4 is confirmed as a pathogen-rich genotype. Eur. J. Protistol. 49, 217–221.

Maghsood, A.H., Sissons, J., Rezaian, M., Nolder, D., Warhurst, D., Khan, N.A., 2005. *Acanthamoeba* genotype T4 from the UK and Iran and isolation of the T2 genotype from clinical isolates. J. Med. Microbiol. 54, 755–759.

Masangkay, F.R., Milanez, G.D., Dionisio, J.D., Ormita, L.A.G.L., Alvarez, A.V., Karanis, P., 2022. Well water sources simultaneous contamination with *Cryptosporidium* and *Acanthamoeba* in east-southeast Asia and *Acanthamoeba* spp. in biofilms in the Philippines. Sci. Total Environ. 837, 155752.

Padua, M.F.F.E., Masangkay, F.R., Alejandro, G.J.D., Milanez, G.D.J., 2023. Detection of *Acanthamoeba* spp. in groundwater sources in a rural area in the Philippines. J. Water Health 21, 138–146.

Putaporntip, C., Kuamsab, N., Nuprasert, W., Rojrung, R., Pattanawong, U., Tia, T., et al., 2021. Analysis of *Acanthamoeba* genotypes from public freshwater sources in Thailand reveals a new genotype, T23 *Acanthamoeba bangkokensis* sp. nov. Sci. Rep. 11, 1729026.

Rivière, D., Szczebara, F.M., Berjeaud, J.M., Frère, J., Héchard, Y., 2006. Development of a real-timePCR assay for quantification of *Acanthamoeba* trophozoites and cysts. J. Microbiol. Methods 64, 78–8327.

Roshni Prithiviraj, S., Rajapandian, S.G.K., Gnanam, H., Gunasekaran, R., Mariappan, P., Singh, S.S., et al., 2020. Clinical presentations, genotypic diversity and phylogenetic analysis of *Acanthamoeba* species causing keratitis. J. Med. Microbiol. 69, 87–95.

Schroeder, J.M., Booton, G.C., Hay, J., Niszl, I.A., Seal, D.V., Markus, M.B., et al., 2001. 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. 39, 1903–1911.

Singh, R.B., Batta, P., 2018. Herpes simplex virus keratitis mimicking *Acanthamoeba* keratitis: a clinicopathological correlation. BMJ Case Rep. 2018 bcr2018226100.

Stamatakis, A., 2015. Using RAxML to infer phylogenies. Curr. Protoc. Bioinformatics 51. https://doi.org/10.1002/0471250953.bi0614s51, 6.14.1-6.14.14.

Thompson, P.P., Kowalski, R.P., Shanks, R.M.Q., Gordon, Y.J., 2008. Validation of real-time PCR for laboratory diagnosis of *Acanthamoeba* keratitis. J. Clin. Microbiol. 46, 3232–3236.

Yera, H., Ok, V., Lee Koy Kuet, F., Dahane, N., Ariey, F., Hasseine, L., et al., 2021. PCR and culture for diagnosis of *Acanthamoeba* keratitis. Br. J. Ophthalmol. 105, 1302–1306.

Zhang, Y., Xu, X., Wei, Z., Cao, K., Zhang, Z., Liang, Q., 2023. The global epidemiology and clinical diagnosis of *Acanthamoeba* keratitis. J. Infect. Public. Health 16, 841–852.