

Article

Investigating Domestic Shower Settings as a Risk Factor for *Acanthamoeba* Keratitis

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Abstract: *Acanthamoeba* keratitis (AK) is a sight-threatening infection of the cornea, which is caused by soil and the waterborne protist *Acanthamoeba* spp. AK most commonly occurs during contact lens (CL) wear. Risk factors for AK have been linked to non-optimal lens hygiene practices and *Acanthamoeba* contamination of domestic water. This study investigated the prevalence of *Acanthamoeba* species in domestic showers in the greater Sydney region, as well as the perception of water contamination of CL as being a risk factor for AK among previous AK patients and their family and friends. Samples from four locations of 13 participants' shower areas were cultured and *Acanthamoeba* 18S rRNA was amplified by PCR, followed by sequencing. Twenty-six responses were received to the online questionnaire. Fifteen water samples (29%, 15/52) contained amoeba that were morphologically classified as *Acanthamoeba* spp. PCR amplification confirmed the presence of *Acanthamoeba* spp. in four samples (8%, 4/52). Three isolates belonged to the T4, and one isolate to the T3 genotype. On the questionnaire survey, 96% (25/26) of respondents believed that water contamination was likely to be a risk factor for *Acanthamoeba* keratitis and 58% rated showering with CL as 'extremely likely' to be a risk factor for AK. *Acanthamoeba* pathogenic genotypes (T3 and T4) in the domestic bathroom water suggest that clinicians should remain vigilant in educating CL wearers about avoiding domestic water contamination of CL.

Keywords: *Acanthamoeba* keratitis; contact lens; domestic shower; domestic bathroom water contamination; prevalence; risk factors

1. Introduction

Acanthamoeba is an amphizoic amoeba with two distinct life stages: an active trophozoite and a dormant cyst. *Acanthamoeba* is found in diverse ecological habitats such as soil, sewage, domestic water supplies, bathtubs, ponds and air conditioning systems [1,2]. It can survive for prolonged periods as stress-resistant double walled cysts, which can excyst to form trophozoites when a suitable condition returns [3]. *Acanthamoeba* is known to cause cutaneous lesions, sinusitis, meningoencephalitis and keratitis [4]. *Acanthamoeba* keratitis (AK) is a potentially blindness-causing, painful and difficult-to-treat corneal infection [5]. Morphologically, *Acanthamoeba* species have been classified into three groups (group I–III), of which pathogenic species are of group II [6]. Based on the 18S rRNA nucleotide sequence, *Acanthamoeba* species are also classified into at least 22 genotypes (T1–T22), with most

keratitis caused by the T4 genotypes, in particular *A. polyphaga* and *A. castellanii* [7,8]. Genotypes T3, T5, T8, T10, T11 and T13 have also been sporadically reported from AK cases [9,10].

The risk factors associated with AK have been investigated extensively since AK was first reported in the United States in 1973 [11]. Use of contact lenses (CL) is the major risk factor for AK, with approximately 90% of reported cases associated with CL wear. Risk factors associated with CL-related AK are swimming or showering with CL, use of tap water while storing or cleaning CL and the unhygienic handling of CL [12–15]. Additionally, most large outbreaks have been related to inadequate disinfection by CL solutions [16–18]. Recent outbreaks have reinforced that the contamination of water with lenses is related to CL-associated AK [19,20]. A community survey conducted in the USA in 2011 revealed that most CL wearers did not consider showering as a risk factor for ocular complications [21].

The incidence of CL-induced AK is approximately 1 to 5 per million CL wearers in the USA and Europe, but it has consistently been around 10 times higher in the UK [14,22]. Such a relatively high incidence in the UK may be attributed to the common practice of rooftop tank-stored tap water and hard tap water, which creates a limescale environment that is ideal for the proliferation of *Acanthamoeba* spp. [14,20]. In a cross-sectional study, Carnt et al. [19] observed that 29% of Sydney household bathroom sinks have been colonized by *Acanthamoeba* spp. Using the same methods, Carnt et al. found a similar proportion in UK household sinks [23].

Despite the low prevalence of AK (10%) among culture proven microbial keratitis [24], the clinical management of AK patients is difficult, with far-reaching health costs. This is partly due to AK often being misdiagnosed as herpetic keratitis, delaying timely treatment at the early stage of infection [25], and partly due to lack of effective antiprotozoal drugs. These issues can often lead to substantial vision loss, even blindness [26]. Poor prognosis of AK is also attributed to transformation into cysts in vivo, which are resistant to medical therapy [25]. Patients with poor outcomes have often experienced long recovery times with more than 31 hospital visits, requiring more than 38 months of hospital follow up and corneal transplants [27]. Even if there is resolution of the disease with anti-amoebic therapy, patients often have visual acuity worse than 6/24 [27].

The current study investigated whether CL wearers who developed AK and their family and friends were aware of the risk of tap water exposure to CL. To account for the possibility of presence of *Acanthamoeba* in domestic water systems, we also assessed its prevalence in domestic shower settings in Sydney, Australia.

2. Methods

2.1. Study Design

This study was approved by the Human Research Advisory Panel (HREAP) at the UNSW, Sydney (HC190359).

The study was divided into two phases. Phase I of the study included a survey of previous AK patients and their family and friends, and phase II evaluated the prevalence of *Acanthamoeba* species in domestic bathroom water samples. A participant information sheet and consent form (PICF) were given to each interested participant in Phase II, and signed consent was obtained before enrolling in the study. In Phase I, implied consent was assumed by the completion of the survey.

2.1.1. Phase I

For the first phase of the study, an anonymous online questionnaire (Qualtrics, Provo, Utah, USA) was created and sent to a closed Facebook group of previous AK patients and their family/friends who may have had particular insight into how AK in shower settings could occur. Participants were asked to rank nine items in terms of likelihood as a risk factor of AK (Appendix A). The items included washing one's face with tap water whilst wearing CL, the use of wet hands to handle CL, indirect exposure to bathroom water whilst wearing CL and the use of CL when showering/bathing. Each practice

was rated out of 5, with response options ranging from 'extremely likely', 'somewhat likely', 'neither likely nor unlikely', 'somewhat unlikely' to 'extremely unlikely'. The idea of the survey was to inform the second part of this study (phase II), which aimed to assess the keratitis-causing genotypes of *Acanthamoeba* spp. in the household bathroom shower water supply of the study participants.

2.1.2. Phase II

The inclusion criteria to enroll in the study were: participants aged ≥ 18 years; ability to swab four locations in the household bathroom; and residing in the Greater Sydney region, as defined by the Australian Bureau of Statistics, 2019 [28]. Participants for phase II were recruited from the public via emails, and study invitation flyers were displayed around the School of Optometry and Vision Science, UNSW, Sydney. Collected water samples were categorized into five different Sydney regions and water delivery plants, based on the postcode of samples received.

2.2. Sample Collection

Water samples were collected between July and August 2019. Each participant was provided with a kit consisting of four sterile polyester fiber-tipped swabs (BD Falcon 220690-Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA), four 15 mL test tubes with caps and detailed instructions to swab four locations (shower head, shower/bathtub drain, vanity drain and shower door/curtain) in the bathroom. Each participant was given a sequential individual study code and the locations coded A-D (1A-D etc.), and each test tube was labelled with the swabbing location and the participant code. Swabs were collected following the guidelines outlined by Kilvington et al. [20]. Participants were advised to briefly swab using the sterile polyester applicator; the outer surface of shower heads; inside the vanity and shower/bathtub drains; and the surface of the shower door/curtain for 10 s, and then to place the swab in a tube with 5 mL of cold water from the shower head. After collection, the tube was fastened tightly and sent to the laboratory for analysis within 24 h. Swab collection was performed in the morning before the use of the bathroom for that day.

2.3. Culture and Microscopic Analysis

Upon receipt, test tubes were vortexed for 20 s, and 1 mL of the sample was applied to the surface of a non-nutrient (1% w/v) agar plate (Oxoid Ltd., Basingstoke, UK) seeded with a suspension of live *Escherichia coli* ATCC 8739 [29]. Plates were left for 15–30 min to absorb the sample water completely, and parafilm-sealed plates were incubated at 28 °C for up to four weeks [4]. The plates were examined using an inverted light microscope (IX73 Inverted Microscope, Olympus Australia Pty Ltd., NSW, Australia) with 40 \times magnification. Amoeba were identified by the appearance of trophozoites or cysts on the plates [6].

2.4. PCR Amplification and Sequencing

Amoebae that were morphologically classified as *Acanthamoeba* were selected for identification by PCR. DNA was extracted using Chelex lysis solution, as outlined previously [30]. Amoeba cells were scraped from the NNA plates using a sterile scraper and mixed with 200 μ L of ice-chilled Chelex lysis solution (10% Chelex in 0.1% of Triton X-100 and Tris buffer of 10 mM/L, pH 8.0). The mixture was heated at 95 °C for 20 min, and cooled in ice followed by mixing for 10 s and centrifugation at 10,000 \times g for 20 s. An aliquot of the supernatant (4 μ L) was used as template DNA for the PCR amplification using genus specific primer pairs (936Fw/1402Rv, 18S rRNA gene) of *Acanthamoeba* spp.; (forward) JDP1 5'-GGCCCAGATCGTTTACCGTGAA-3' and (reverse) JDP2 5'-TCTACAAGCTGCTAGGGGAGTCA-3' which gives a 450-bp PCR amplicon. Each 50 μ L of PCR mixture comprised 25 μ L of DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, Walsham, MA, USA), 2.5 μ L of each primer (10 μ M) and 16 μ L of ddH₂O. The PCR conditions were as follows: denaturation at 95 °C for 5 min, followed by 35 cycles of amplification (94 °C for 30 s, 56 °C for 30 s and 72 °C for 45 s), final elongation at 72 °C for 10 min, and

hold at 4 °C [19,31]. Amplicon bands were visualised using gel electrophoresis. PCR-positive samples were sent to the Ramaciotti Centre for Genomics at UNSW, Sydney for Sanger sequencing using primer pairs (forward) 892C 5'-GTCAGAGGTGAAATTCTTGG-3' and (reverse) JDP2 5' TCTCACAAGCTGCTAGGGGAGTCA-3' [32].

2.5. Phylogenetic Reconstruction

To gauge the genetic relationship between PCR-positive isolates with other bathroom isolates of the Sydney region from our previous study [19], a phylogenetic tree was constructed using the maximum likelihood method with 100 bootstraps using PhyML v 3.2 [33] (LIRMM, UMR France).

3. Results

3.1. Survey Questionnaire (Phase I)

A total of 26 responses from participants aged ≥ 18 years were collected from the online questionnaire posted to the AK Facebook Group. The two activities in the bathroom perceived as 'extremely likely' to cause AK were 'bathing whilst wearing CL' and 'indirect water exposure from showering whilst wearing CL' (Figure 1, Table S1). Twenty-five participants (96%) rated the indirect water exposure from showering whilst wearing CL as either 'extremely likely' or 'somewhat likely', while one participant (4%) rated it 'neither likely nor unlikely'. The other two behaviors that were observed as 'somewhat likely' were using wet hands to handle CL (46%) or inserting and/or removing lenses in the bathroom (27%).

3.2. Detection of *Acanthamoeba* spp. in Collected Samples (Phase II)

Thirteen participants were recruited to collect water samples from the four locations, resulting in 52 water samples in total. Different morphological forms of amoeba trophozoites or cysts were observed. Fifty-two cultures were classified into five groups: 'clearly' *Acanthamoeba* spp.; 'likely'; 'possible'; 'unable to observe'; and 'highly unlikely' for either the trophozoite or cyst forms. In total, fifteen samples (15/52) were identified as being morphologically similar (clearly, likely and possible combined) to *Acanthamoeba* spp. The vanity drain had the highest rate (8/15, 53.3%) of its total samples being morphologically positive for *Acanthamoeba* spp., followed by shower/bath drain (6/15, 40%) and shower door/curtain (1/15, 6.7%). No samples from the shower head were classified as *Acanthamoeba* (Figure 2).

Upon nucleotide BLAST searches, one sample (11B) from a shower drain and two samples from bathroom vanity drains (2C, 8C) were identified as *A. castellanii* T4. A sample from the shower curtain/door (3D) was identified as *A. castellanii* T3 (Figures 2–4). Three isolates of the current study were phylogenetically close to those which had been isolated in 2017–2018 in our previous study using the same methods [19]. One isolate (3D) which belongs to the T3 genotype was a phylogenetic outlier in this analysis (Figure 4). A photograph of samples (2C, 3D, 8C and 11B) with PCR amplicon bands produced using *Acanthamoeba* genus specific primer pair 936Fw/1402Rv is mentioned in Figure S1 and the sequence data file is included in supplementary file.

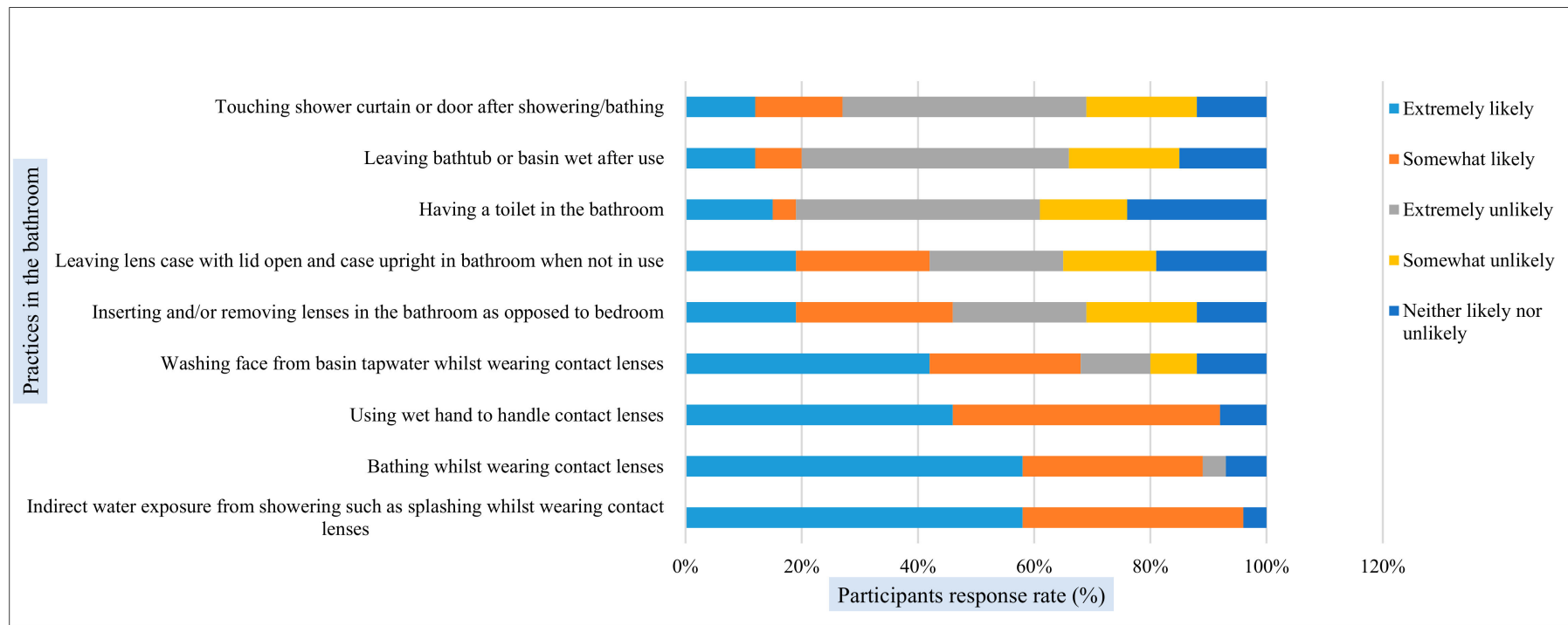


Figure 1. Participants’ perception of the likelihood of each contact lens (CL) practice in the bathroom as a risk factor for *Acanthamoeba* keratitis (AK).

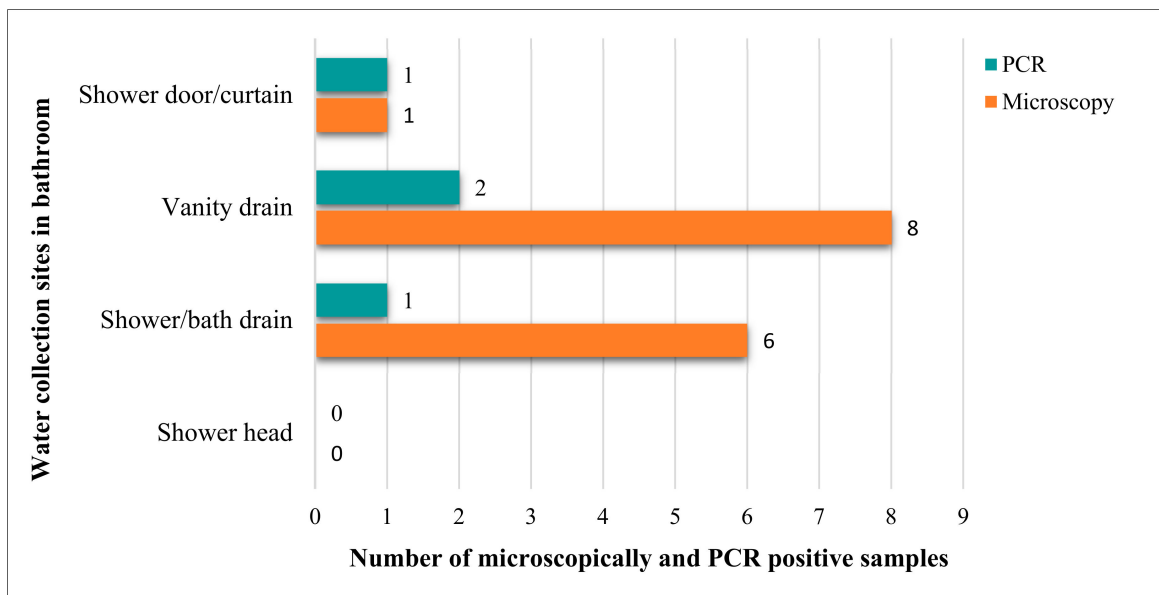


Figure 2. Number of morphologically and PCR-positive samples for each location of the bathroom.

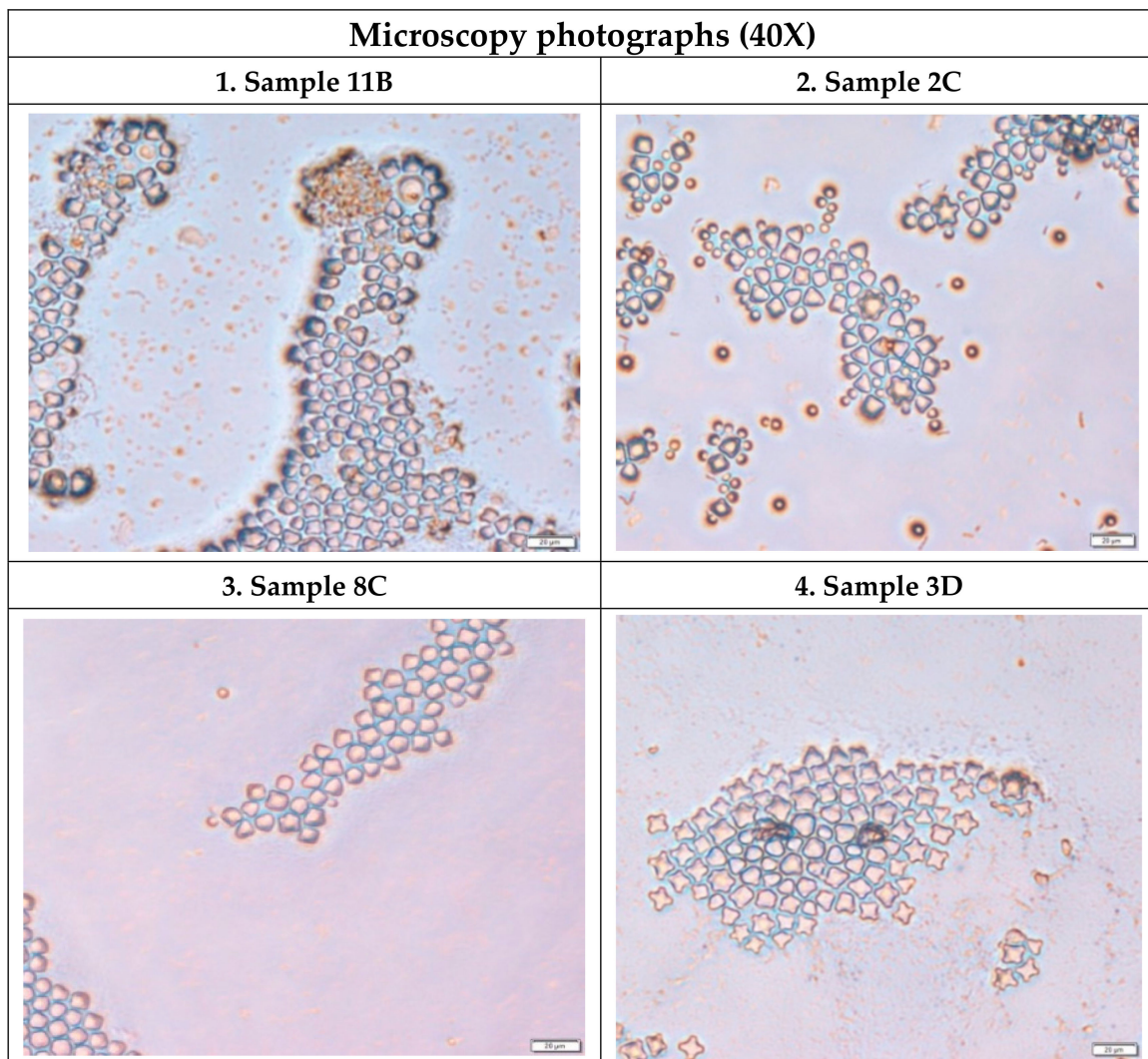


Figure 3. Microscopy photographs (40X) of the four PCR-positive samples (4/52, 7.7%).

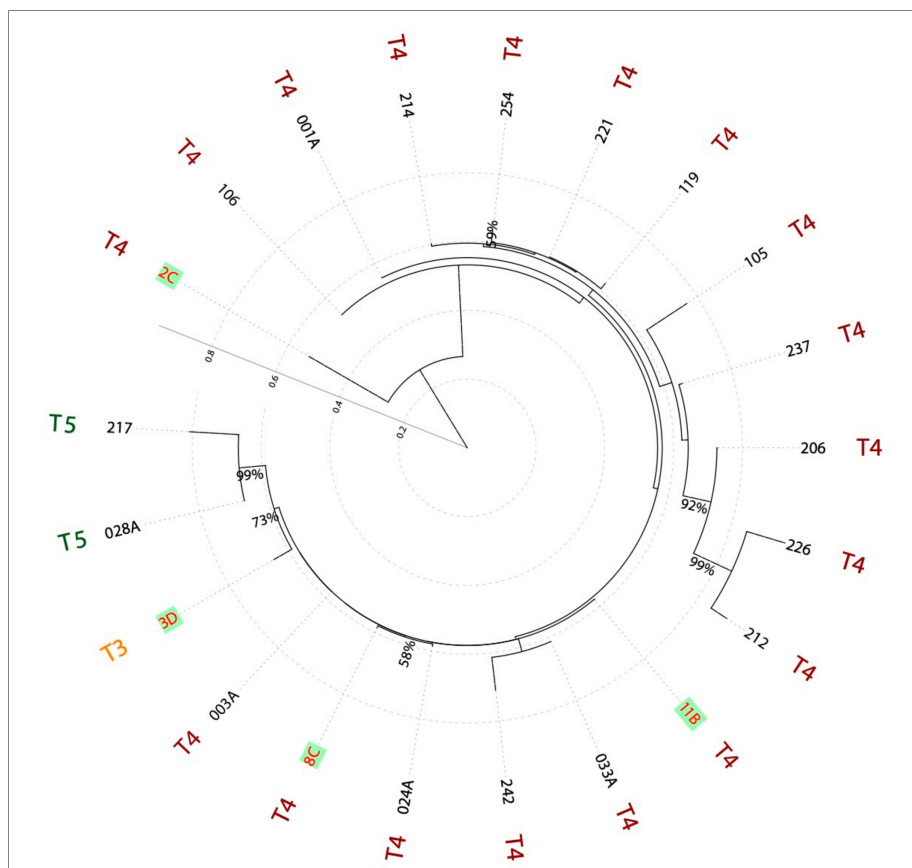


Figure 4. Radial view of phylogenetic relationships between four isolates of this study (highlighted in green) and other bathroom *Acanthamoeba* isolates from the previous study [19]. Tree scale (nucleotide substitution per 100 bp) is shown internally, and a bootstrap value of more than 50 is shown in each node. Numbers in the ring denote isolate designation and outmost values denote genotype (T3, T4 and T5 genotypes). The tree was visualized using iTol v5 [34].

4. Discussion

The prevalence of *Acanthamoeba* spp. in the domestic water supply is an important public health issue in understanding the potential risk of developing AK in a CL-wearing population. CL-induced AK is associated with the contamination of domestic water with *Acanthamoeba*, and the use of CL during showering and/or swimming is a major risk factor of AK [20,35,36]. As 90% of AK cases are associated with CL wear [5], *Acanthamoeba* infection probably begins with the water contamination of CL. Indeed, various forms of water exposure to CL have been reported to be substantial risk factors of AK [37,38].

Acanthamoeba spp. are frequently reported from household tap water, particularly in endemic or potentially endemic regions [35,36]. In a study reported from the metropolitan area of Mexico City, 70% (19/27 samples) of domestic tap water was positive for *Acanthamoeba* spp., and the highest number of amoeba isolates were from roof tanks and water cisterns [28], although most of the amoeba isolates were considered to be non-pathogenic. We previously reported that 29% of isolates were morphologically similar to *Acanthamoeba* spp. in bathroom vanity drains in the greater Sydney region across two seasons (30% summer, and 28% winter) [19]. This is in line with the current study, where 15 samples (15/52, 29%) were morphologically similar to *Acanthamoeba* species. Of these, however, only four were confirmed as *Acanthamoeba* spp. on 18S rRNA PCR amplification. On nucleotide sequence analysis, three isolates were identified as T4 and one isolate as T3 genotypes of *Acanthamoeba* spp. Low PCR positivity might be due to the presence of other free living amoeba (FLA) in the studied samples that were morphologically similar to *Acanthamoeba* [39].

Colonization of *Acanthamoeba* was higher in the vanity drain (53.3%), followed by the shower/bath drain (40%) and shower door/curtain (6.7%). There were no morphological traces of *Acanthamoeba*-like trophozoites or cysts in swabs collected from the showerheads. Participants of the study did not screw off the shower heads, and only swabbed the surface. Therefore, the biofilm inside the showerhead might not have been collected. Furthermore, the fast flow of water through shower heads may have dislodged *Acanthamoeba* and prevented colonization on the surface of the shower head. A study reported from the UK by Carnt et al. [23] observed the lowest rate of *Acanthamoeba* isolation in kitchen and bathroom spouts, as compared to drains, which is thought to be due to spout filters, which prevent biofilm being dislodged by the swabbing procedure [20]. High prevalence (79%) of *Acanthamoeba* spp. and other FLA were observed in the water of bathroom shower heads from the residents of two counties of Ohio, the USA, where detection of amoeba was higher in swabs of biofilm compared to only collected water [39]. This highlights that thorough sampling of shower heads both inside out is important to determine the actual prevalence of *Acanthamoeba* in shower settings.

The water supply in bathrooms of AK patients are more likely to be contaminated with FLA than the bathroom water of asymptomatic CL wearers in the UK [23]. Among 100 households, bathroom sink water analyzed from a range of locations in Hong Kong, 10% of samples were positive for *Acanthamoeba*; higher contamination of water with amoeba was observed in samples received from urban areas and older buildings [40]. Residents with a hard water supply in the UK are three times higher at risk of AK infection, compared to residents with a soft water supply [14]. The presence of limescale/calcium scale (CaCO_3) in hard water may deposit inside the water pipes, and provide an excellent environment for bacterial biofilm where *Acanthamoeba* spp. proliferate more efficiently as it feeds on bacteria [19]. The metropolitan drinking water supply of Sydney contains 2–70 mg/L of CaCO_3 , so it is considered to be a soft water region [41]. Therefore, it may be likely that the water supply in areas with “harder” water such as Brisbane (46–150 mg/L) and Adelaide (69–151 mg/L) are at greater risk of containing *Acanthamoeba* in their domestic water supplies [42,43]. However, this warrants further investigation.

Similar to other environmental studies where the genotype T4 of *Acanthamoeba* spp. was reported as the predominant genotype [44–46], the current study found that 3/4 (75%) of sequenced strains were T4 genotype irrespective of the sample site in bathrooms. Additionally, one isolate from the shower curtain/door was identified as *A. castellanii* T3. The genotype T3 has also been associated with AK [47,48], although it is less common than T4. In vitro studies have shown T4 genotype of *Acanthamoeba* exhibits higher binding capacity to human corneal epithelial cells and produces greater cytotoxicity [48,49], while other studies have observed that non-T4 genotypes have shown more pathogenic effects, and are more resistant to multipurpose CL cleaning solutions (MPS) than virulent T4 isolates [50,51]. Three isolates of the current study were phylogenetically close to those which were isolated in 2017–2018 [19] from the domestic water of the Sydney region. This shows the possibility of the persistence of similar genotypes of *Acanthamoeba* spp. in the domestic water supply of Sydney. The relatively high prevalence of *Acanthamoeba* in domestic water means that awareness of patients to not contaminate their CL or lens cases with tap water is important and may help to reduce the incidence of AK. The two activities ‘bathing whilst wearing CL’ and ‘indirect water exposure from showering whilst wearing CL’ were most often perceived as ‘extremely likely’ to cause AK by the study participants. This is a good indicator that education and reinforcement of messages about water has had an impact on people who have had AK and their families and friends. Whether the same is true in the general population of CL wearers in Sydney requires further research.

5. Conclusions

Despite low cases of AK in the greater Sydney region, potentially pathogenic *Acanthamoeba* spp. were observed in bathroom water, particularly in shower and vanity drains. The two practices ‘bathing whilst wearing CL’ and ‘indirect water exposure from showering whilst wearing CL’ in the bathroom were perceived as ‘extremely likely’ to cause *Acanthamoeba* keratitis by the AK patients and their family and friends. The awareness of these practices as being problematic is important, as it may help to

reduce the likelihood of CL wearers developing AK. Clinicians should make wearers aware of good CL practice, including avoiding water contamination of CL and storage cases.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4441/12/12/3493/s1>, Figure S1: Image of samples (2C, 3D, 8C, and 11B) with PCR amplicon bands produced using *Acanthamoeba* genus specific primer pair 936Fw/1402Rv. Amplicon bands were observed using the Gel Doc XR + Gel Documentation System, Bio-Rad laboratories, CA, USA: Table S1. Percentage of participants responses to nine online survey questions of likelihood as a risk factor of *Acanthamoeba* keratitis (AK); Nucleotide sequence file.

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Abbreviation

AK	<i>Acanthamoeba</i> keratitis
BLAST	Basic local alignment search tool
CL	Contact lens
FLA	Free living amoeba
MPS	Multipurpose solution

Appendix A. Online Questionnaire Posted on *Acanthamoeba* Keratitis Facebook Group

26/11/2019 Qualtrics Survey Software

Default Question Block

After having read the consent form, do you give consent for your responses to be used in research? Your responses will remain anonymous for your confidentiality.

Yes
No

Block 1

Please rank the likelihood for each options as a risk factor for *Acanthamoeba* Keratitis: (grading scale for each option ranging from 'extremely likely', 'somewhat likely', 'neither likely nor unlikely', 'somewhat unlikely' and 'extremely unlikely' in one table).

	Extremely likely	Somewhat likely	Neither likely nor unlikely	Somewhat unlikely	Extremely unlikely
Indirect water exposure from showering such as splashing whilst wearing contact lenses.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Using wet hands to handle contact lenses.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Inserting and/or removing lenses in the bathroom as opposed to bedroom.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Leaving lens case with lid open and case upright in bathroom when not in use.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Washing face from basin tapwater whilst wearing contact lenses.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Having a toilet in the bathroom.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Bathing whilst wearing contact lenses.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Leaving bathtub or basin wet after use.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Touching shower curtain or door after showering/bathing.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Figure A1. The survey asked about the beliefs of behaviours in bathroom setting as risk factors for developing *Acanthamoeba* keratitis.

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