The prevalence and seasonal variation of *Acanthamoeba* in domestic tap-water in greater Sydney region, Australia

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Running title: Acanthamoeba in domestic tap-water in Sydney

Abstract:

Purpose: To assess the prevalence of free-living *Acanthamoeba* in domestic tap-water in the greater Sydney region in New South Wales state, Australia, and determine any seasonal variation in prevalence.

Methods: A sample size was calculated as 43 to give significance at 5% with 90% power. To account for 20% drop out, 54 participants were enrolled following approval from Institutional Human Research Ethics Committee. The participants collected bathroom tap-water samples from their homes using an instructional kit. The samples were cultured by inoculating onto a non-nutrient agar plate seeded with *Escherichia coli* and incubation at 32˚C for 2 weeks. The plates were morphologically examined for the presence of free-living *Acanthamoeba* using an inverted light microscope. Partial sequence of 18S rRNA of 20 culture positive samples were obtained to assess genotypes. Each participant collected samples two times over one year, once in summer and again in winter. The association between sampling seasons was analysed with chi-square test.

Results: A total of 97 samples were collected over the two collection periods, with 28.57% of samples morphologically classified as *Acanthamoeba*. The summer period yielded 16 of 54 (29.63%) samples classified as *Acanthamoeba*, while the winter period yielded 12 of 43 (27.90%) samples classified as *Acanthamoeba*. There was no statistically significant difference (p=0.85) between the prevalence of free-living *Acanthamoeba* in summer compared to winter. Phylogenetic analysis showed that 15 of 20 (75%) isolates belonged to genotype T4, the most frequently isolated genotypes of *Acanthamoeba* Keratitis.

Conclusion: The prevalence of free-living *Acanthamoeba* characterised morphologically in domestic tap-water of the greater Sydney region was higher than expected, especially considering the low incidence of *Acanthamoeba* keratitis in Australia. We did not however find variation between seasons. While the T4 genotype was most common, Sydney-based practitioners must always consider *Acanthamoeba* as a possible causative organism in cases of microbial keratitis regardless of the season.

Introduction

Acanthamoeba is a free-living protozoan capable of causing *Acanthamoeba* keratitis, a rare infection of the cornea that could lead to blindness (Clarke & Niederkorn, 2006; Marciano-Cabral & Cabral, 2003). *Acanthamoeba* species are present in a large proportion in different environments. Having the capacity to form resilient cysts, this organism is able to survive extreme environmental conditions including residual chlorine concentration of household water (Schuster, 2002). This may be the reason that *Acanthamoeba* species have been reportedly isolated from human-made environments such as swimming pools, hot tubs, tap water, shower water and the atmosphere (De Jonckheere, 1991; Kingston & Warhurst, 1969; Seal, Stapleton, & Dart, 1992). The incidence of *Acanthamoeba* keratitis is on the rise and more than 85% infections are associated with contact lens wearers (Ku, Chan, & Beckingsale, 2009; Stehr-Green et al., 1987; Stehr-Green, Bailey, & Visvesvara, 1989). The risk factor includes exposure of contact lenses to the organism by activities such as swimming or showering while wearing lenses and poor lens hygiene. This has raised the concern that source water contamination could be associated with *Acanthamoeba* keratitis (Kilvington et al., 2004).

The incidence of *Acanthamoeba* keratitis is 17.53-21.14 per million contact lens wearers compared to 1.26 per million non-contact lens wearers recorded in the UK (Radford, Minassian, & Dart, 2002). In comparison, the incidence of *Acanthamoeba* keratitis in

Australia is relatively low, at 5 per million contact lens wearers (Schaumberg, Snow, & Dana, 1998). Various species of *Acanthamoeba* are associated with keratitis. Based on morphological differences, acanthamoebas are classified into three groups (I, II and III) (Pussard & Pons, 1977). Group II acanthamoebas constitute strains that are most commonly isolated form environments and have been most commonly identified as causative organisms for *Acanthamoeba* keratitis (Maciver, Asif, Simmen, & Lorenzo-Morales, 2013). Group III strains are less frequently isolated from human infections compared to Group II and Group I is not a known cause of *Acanthamoeba* keratitis (Booton, Visvesvara, Byers, Kelly, & Fuerst, 2005; Lorenzo-Morales et al., 2015; Maciver et al., 2013; Qvarnstrom, Nerad, & Visvesvara, 2013). Acanthamoebas are also genetically grouped based on ribosomal RNA gene (18S rRNA) (Gast, Ledee, Fuerst, & Byers, 1996). To date, at least 20 genotypes (T1-T20) of acanthamoebas have been identified (Corsaro, Walochnik, Köhsler, & Rott, 2015). Of the various genotypes, the T4 genotype which includes *A. castellanii*, *A. polyphaga* and *A. culbertsonii*, is most commonly associated with *Acanthamoeba* keratitis (Booton et al., 2009; Gatti et al., 2010; Ledee et al., 2009; Sharma, Pasricha, Das, & Aggarwal, 2004; Zhao, Sun, Zhao, & Xie, 2010). Understanding of abundance of pathogenic or virulent genotypes in water sources may help to predict potential risk of *Acanthamoeba* keratitis.

Various studies have demonstrated a seasonal variation in the incidence of *Acanthamoeba* keratitis and in the concentration of free-living *Acanthamoeba* in water, with higher rates during warmer seasons (Kao et al., 2013; Kyle & Noblet, 1986, 1987; Mathers, Sutphin, Lane, & Folberg, 1998; McAllum et al., 2009; Taher, Meabed, Abdallah, & Abdel Wahed, 2018; Yoder et al., 2012). However, it should be noted that the latter studies examined outdoor bodies of water (which are susceptible to external factors that can potentially skew results attained) rather than domestic water, which has been implicated as the main source of

contact lens associated *Acanthamoeba* keratitis. Findings from the preliminary study conducted in the UK shows 27% of samples collected from drains were found to be positive for *Acanthamoeba* (Carnt, Kilvington, Connor, & Dart, 2018). This has therefore driven this investigation of *Acanthamoeba* in domestic water in Sydney. No other studiy has assessed the prevalence of free-living *Acanthamoeba* in Australia, thus it is difficult to ascertain the population's risk of developing *Acanthamoeba* keratitis. Further, it is debatable if findings regarding seasonal variations of *Acanthamoeba* are applicable in Australia, considering the climate and temperature difference to the study locations stated above. Therefore, this study aimed to determine the prevalence of free-living *Acanthamoeba* in domestic tap-water in greater Sydney region using morphological assay and 18S rRNA typing including presence of seasonal variation.

Methods:

Sample Collection

A total of 54 participants residing in the Greater Sydney region were included in this study. The research protocol received approval from institutional Human Research Ethics Committee (HC180048) and followed the amended version of Declaration of Helsinki (2013). Written informed consent was received from participants before study initiation. Tap water was collected from household bathrooms following guidelines described earlier by Kilvington *et al*. (Kilvington et al., 2004). Briefly, each participant was provided with a sampling pack containing a sterile polyester-tipped applicator, a sterile screw-cap test tube, written instructions including photographs, and a questionnaire which included questions on the suburb, date and time of sample collection, and the date and time that samples were returned to researchers. Participants were requested to swab the inside of their bathroom sink drain or overflow for 10 seconds with the applicator, place the swab into the test tube, fill the tube with 5mL cold tap-water, then fasten the test tube cap tightly. This sample collection

was conducted in the morning before the bathroom tap was used for the day. The samples and completed questionnaires were returned within 24 hours of collection for laboratory analysis.

Culture and Morphological Assessment

Test tubes containing samples were vortexed and 300µL of each sample was inoculated onto a separate 1.4% non-nutrient agar (NNA) plate preseeded with 100µL of living *Escherichia coli* and incubate at 32˚C (Kilvington, Larkin, White, & Beeching, 1990). After incubation for 3-4 days, plates were examined daily for two weeks using an inverted microscope. Organisms matching the morphological appearance of *Acanthamoeba* trophozoites or cysts based on Khan's illustrations (Khan, 2009) were photo-documented using the Olympus IX71 inverted microscope with a 40x objective lens (Olympus America, Melville, NY) and Olympus DP80 digital camera system. Photo-documented cysts were classified into three groups according to their likelihood of being *Acanthamoeba*, based on the known morphology of *Acanthamoeba* . Morphologically, trophozoites appear as oval or ellipsoid structures typically 15-45µm, and are characterized by the presence of filiform pseudopodia and contractile vacuoles, with a rate of movement of approx. 0.8µm/s (Fig 1) (Lorenzo-Morales et al., 2015). The cyst form of *Acanthamoeba* is typically 12-25µm, and is characterized by the presence of a double-wall with several points of contact between the endocyst and ectocyst, which may be stellate or star-shaped, polygonal, or round (Fig 2) (Lorenzo-Morales et al., 2015; Siddiqui & Khan, 2012). Cysts matching all the above features were classified as 'likely' being *Acanthamoeba*. Cysts lacking one of the above features were classified as 'possibly' being *Acanthamoeba*, while all other observed cysts were classified as 'unlikely' to be *Acanthamoeba*. Cysts classified as 'likely' or possible' were also classified into morphological groups I-III, as per Pussard and Pons' classification scheme (Pussard & Pons, 1977) described elsewhere (Lek-Uthai, Passara, & Roongruangchai, 2009; Lorenzo-Morales et al., 2015).

PCR assay, 18S rRNA sequencing and sequence homology analysis

The DNA from cysts of *Acanthamoeba* was isolated using Chelex resin (MB Chelex-100 resin; Bio-Rad Laboratories, Hercules, CA, USA) following the method described by Kilvington et al. (Iovieno, Miller, Lonnen, Kilvington, & Alfonso, 2011). Briefly, cysts were picked from the growth culture and mixed with 200µL of ice-cooled Chelex solution (Chelex 10% [wt/vol] in 0.1% Triton X-100 and 10 mM Tris buffer [pH 8.0]). The suspension was centrifuged at 10,000 g for 10s and then heated at 95°C for 20 min followed by cooling on ice and finally centrifuged at 10,000 g for 20 s. 4 µL of supernatant was used for PCR amplification using *Acanthamoeba* genus-specific primers; forward JDP1 5'-GGCCCAGATCGTTTACCGTGAA-3' and reverse JDP2 5'-TCTCACAAGCTGCTAGGGGAGTCA-3' which gives a 450-bp product. PCR amplification was carried out in 50µl of final reaction mix using DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, US) with temperature cycle as follows; initial denaturation at 95°C for 5 min, followed by 35 cycles of amplification (94°C for 30 sec min, 56°C for 30 sec and 72°C for 45 sec) with final elongation at 72°C for 10 min. PCR products were examined on 1% agarose gel and positive samples were sent to the Ramaciotti Centre for genomics (The University of New South Wales, Australia) for Sanger Sequencing using primer 892c 5'-GTCAGAGGTGAAATTCTTGG-3' (forward) and JDP2 5'-TCTCACAAGCTGCTAGGGGAGTCA-3' (reverse) (Behera, Satpathy, & Tripathi, 2016). Forward and reverse nucleotide sequences were assembled using CAP3 (Huang & Madan, 1999) and were used for BLAST searches against the NCBI database to examine sequence similarities with available genotypes of *Acanthamoeba*. Finally, sequences were aligned using CLUSTALW and the phylogenetic tree was reconstructed using Kimura parameter

with 1000 bootstrap using MEGA7 (Kumar, Stecher, & Tamura, 2016). Tree was visualised and represented using iTOLv4 (Letunic & Bork, 2019).

Statistical analysis

Based on figures from a similar study that investigated seasonal trends of free-living *Acanthamoeba* (Kao et al., 2013), it was determined that a sample size of 43 participants was necessary to achieve a statistically significant difference in the prevalence of *Acanthamoeba* in summer and winter at the 90% power and 5% significance level. The Pearson Chi-square test was used to assess whether there was a statistically significant difference in the prevalence of free-living *Acanthamoeba* in summer and winter.

Results

Detection rate of free living Acanthamoeba

A total of 97 tap-water samples (54 from the summer period and 43 from the winter period) were examined. The 20% drop-out rate during winter was due to participants' lack of availability. Temperature for summer ranged between 14.0˚C and 24.9˚C and temperature for winter ranged between 4.6˚C and 17.5˚C. Different morphological forms of cysts were observed (Fig 3). However, samples classified as 'likely' was used to estimate the prevalence of *Acanthamoeba* in tap water (Lorenzo-Morales et al., 2015; Siddiqui & Khan, 2012). As shown in Fig 4, the rates of contamination of tap water by *Acanthamoeba* was 29.63% in summer and 27.90% in winter. There was no statistically significant difference ($p=0.85$) in the prevalence of free-living *Acanthamoeba* in summer compared to winter.

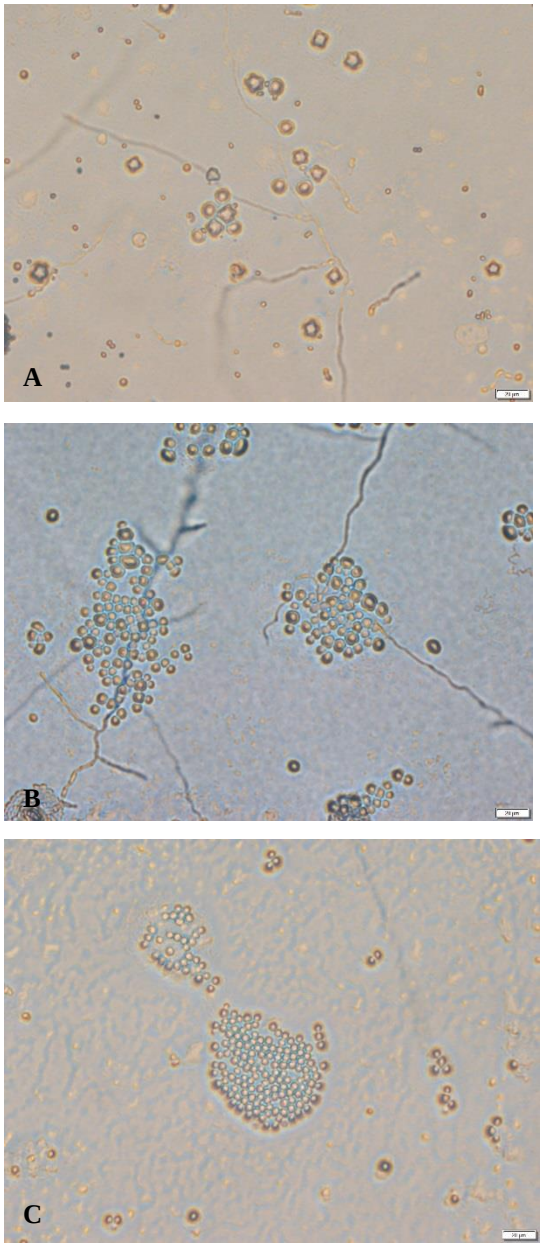

Figure 3 Morphologies of *Acanthamoeba* cysts observed in this study, classified on the basis of (Lorenzo-Morales, Khan, & Walochnik, 2015; Siddiqui & Khan, 2012)**. (A)** 'likely'; **(B)** 'possible'; **(C)** 'unlikely'

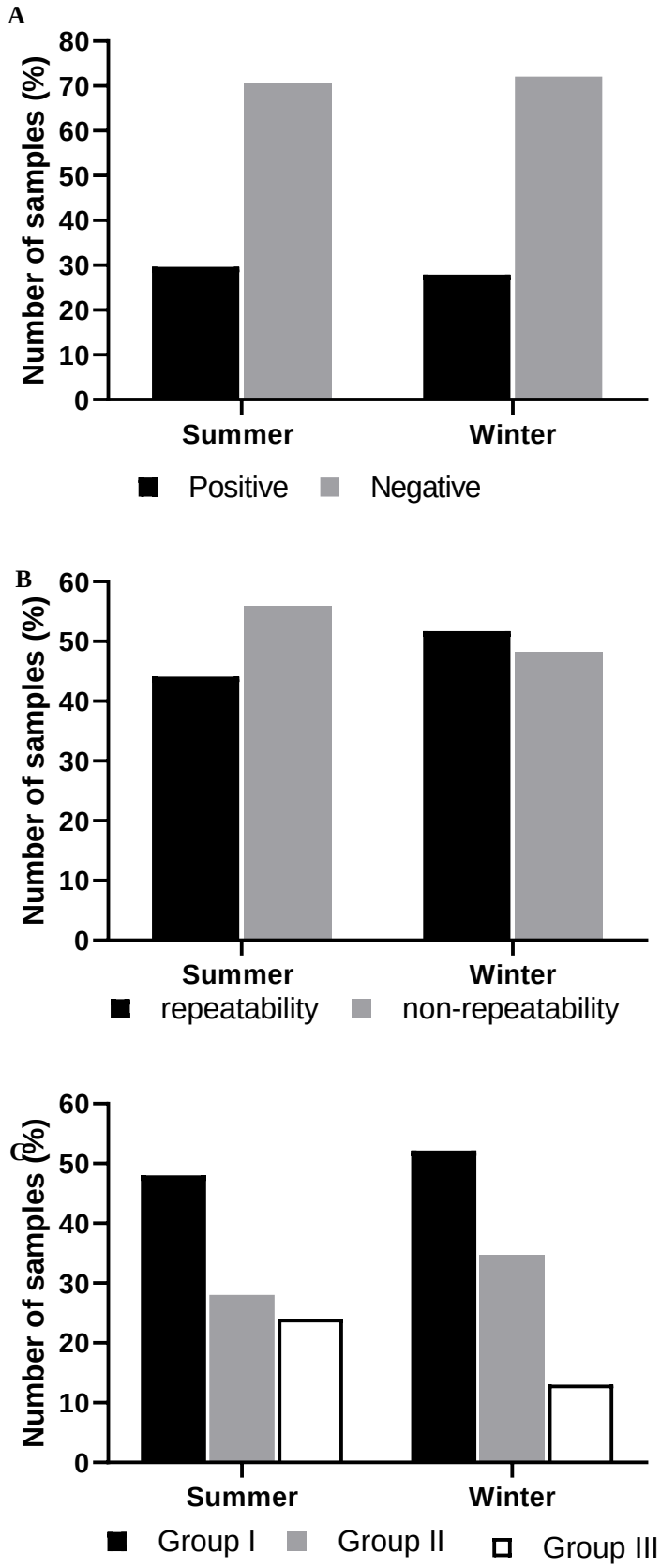

Figure 4 Prevalence of *Acanthamoeba* in summer and winter (A) Percentage of Acanthamoeba positive samples in summer and winter. (B) Percentage of occurrence of

Acanthamoeba positive samples repeatedly in the same household in summer and winter. (C) Percentage of samples classified in to Pussard and Pon's group.

To examine the repeatability of detection of *Acanthamoeba* in the same household during winter and summer, we considered all three possible forms of cysts as mentioned in methods. Approximately half of the household bathrooms were positive for 'potential' *Acanthamoeba* both during summer and winter (Fig 4B), indicating the possibility of persistent contamination of tap water. Based on Pussard and Pon's classification scheme, 28% of summer and 34.78% of winter positive samples were classified as Group II (Fig 4C), which have been most commonly identified as causative organisms for *Acanthamoeba* keratitis (Lorenzo-Morales et al., 2015).

Genotypic analysis and phylogenetics

Partial nucleotide sequences of 18S rRNA of randomly selected 20 samples (9 summer and 11 winter) were obtained and compared with the NCBI database to confirm genus using BLASTn searches. All the sequences had high similarities (95%-100%) with genus *Acanthamoeba*. Nucleotide sequences of T3, T4 and T15 were included as references in the phylogenetic reconstruction (Di Cave et al., 2014) using the Neighbor-Joining method (Saitou & Nei, 1987). Fifteen isolates of this study, which included both summer and winter samples were placed within a clade of previously identified strains AcaL4 and AcaL2 of genotype T4 (Fig 5). One isolate was loosely associated with these genotypes and four isolates formed a separate clade. The isolates of the separate clade had shown 100% identity with *A. lenticulata* (T5-genotype).

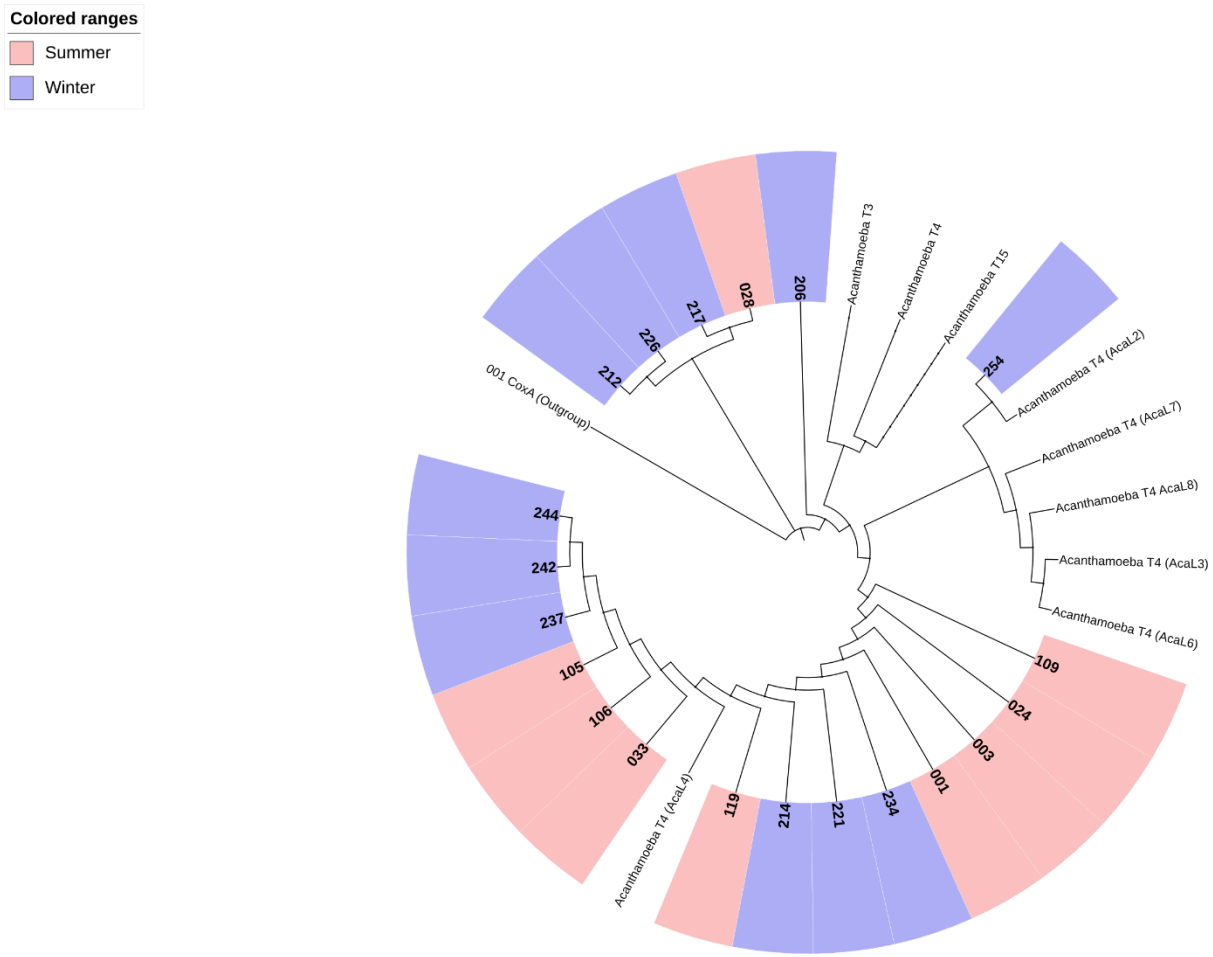

Figure 5 Radial view of Neighbor-joining tree showing the genetic relationships among 20 *Acanthamoeba* isolates examined in this study, based on reference sequences of strains of T3, T4, T15 genotypes from NCBI database.

Discussion

The prevalence of free-living *Acanthamoeba* in domestic water systems in Australia has not so far been reported. Contaminated domestic water is known to be a source of *Acanthamoeba* spp., which is associated with contact-lens related *Acanthamoeba* keratitis (Joslin et al., 2006; Kilvington et al., 2004; Radford et al., 2002). We began this work with the aim to determine the prevalence of *Acanthamoeba* in domestic tap-water in Sydney and have used morphological assay and 18S rRNA typing to identify isolates, with a particular focus on seasonal variation in this prevalence. Our study yielded a similar detection rate of free-living *Acanthamoeba* in domestic water supplies to Kilvington et al.'s study conducted in the UK

(Kilvington et al., 2004), there was no seasonal variation in this prevalence in summer compared to winter and the majority of isolates were genotypically similar to known pathogenic genotype T4 of *Acanthamoeba* (Booton et al., 2009; Booton et al., 2005; Di Cave et al., 2014).

Although the prevalence of *Acanthamoeba* spp. in Sydney's household water supplies was found similar to the UK's supplies, the prevalence of *Acanthamoeba* keratitis in Australia is significantly less than the UK and other developed countries (Kilvington et al., 2004) (Di Cave et al., 2014; Joslin et al., 2006) (Stehr-Green et al., 1989). This discrepancy in the prevalence of *Acanthamoeba* keratitis may reflect differences in virulent phenotype or genotype of *Acanthamoeba* in Sydney compared to the UK. To examine this, we classified cultured samples both morphologically and genotypically. Based on Pussard and Pon's classification system, 15 (32%) cysts identified over the two collection periods appeared to belong to Group II, which have been most commonly identified as causative organisms for *Acanthamoeba* keratitis (Lorenzo-Morales et al., 2015). In contrast, many studies have found that T4 *Acanthamoeba* is predominantly environmental and clinical genotypes including keratitis (Booton et al., 2009; Booton et al., 2005; Gatti et al., 2010; Khezri et al., 2016). This study observed that 75% of sequenced isolates belonged T4 genotype regardless of period of sample collections. This result contradicts the finding of morphological grouping and supports the observations that morphological classification could be ambiguous (Khezri et al., 2016; Lorenzo-Morales et al., 2015). In addition, phylogenetic analysis revealed that five isolates (out of 20 sequenced samples) were in different clade than T4. Sequence analysis of isolates of this clade showed that they belonged to T5 genotypes, which include *A. lenticulata* and can be associated with human infections including keratitis (Booton et al., 2009; Schroeder et al., 2001).

In this study, there was no significant difference between the prevalence of *Acanthamoeba* in summer compared to winter. This is in contrast to previous studies which have reported a greater incidence of *Acanthamoeba* keratitis, and greater prevalence of free-living *Acanthamoeba* in outdoor bodies of water, in summer compared to winter (Kao et al., 2013; Kyle & Noblet, 1986, 1987; Mathers et al., 1998; McAllum et al., 2009; Taher et al., 2018; Yoder et al., 2012). It is difficult to assess if the higher incidence of *Acanthamoeba* keratitis reflects a higher prevalence of free-living *Acanthamoeba* in summer or the phenomenon is just an effect of human behaviour during summer e.g. swimming with contact lenses, or a combination of both. One study has illustrated a direct correlation between concentration of *Acanthamoeba* in pond water and incidence of *Acanthamoeba* keratitis during summer (Mathers et al., 1998). It is also important to bear in mind that most studies investigating seasonal differences in free-living *Acanthamoeba* prevalence have been conducted on outdoor bodies of water, which are susceptible to external factors such as rainfall and turbidity. This can encourage resuspension of organisms, resulting in a falsely higher presence of *Acanthamoeba*. In addition, there have been reports of an increased prevalence of *Acanthamoeba* keratitis cases after flooding where water supplies have been contaminated (Meier et al., 1998). These studies are perhaps less relevant to cases of *Acanthamoeba* keratitis in contact lens wearers, as the causative organism usually originates from domestic water sources. Further studies with larger sample sizes from other regions in Australia are required to confirm the results of seasonal variation observed in this study.

This study observed different morphological forms of cysts, which were ‘unlikely’ to be *Acanthamoeba* and excluded from our analysis. Given that other free-living amoebae are also prevalent in water bodies (Kilvington et al., 2004), further study of mitochondrial *cox* gene sequence will require to differentiate the species. This study found the possibility of persistent contamination of tap water in half of the studied household bathrooms in both

seasons which could be associated with water supply systems including residual chlorine concentration (Schroeder et al., 2001; Taher et al., 2018). Therefore, further study should also aim at correlating isolation of *Acanthamoeba* with the factors that could affect water supply system.

The high rate of Acanthamoeba detection in tap-water highlights that practitioners should remain vigilant in enforcing to patients the importance of avoiding contamination of contact lenses with water. Greater Sydney-based practitioners must always consider *Acanthamoeba* as a possible causative organism in cases of microbial keratitis regardless of the season, especially if infections are not responding well to antibiotics or present with signs and symptoms which are not characteristic of a bacterial infection.

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