

## Prevalence of *Acanthamoeba* and other naked amoebae in South Florida domestic water

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### ABSTRACT

The purpose was to identify the prevalence of naked amoebae in tap water in south Florida to ascertain the risk of amoebal infections of the cornea in contact lens wearers.

Over the course of a 2-year period, water samples were collected from sites throughout Broward, Palm Beach, and Dade counties, Florida. The presence of amoebae in samples was based on an enrichment cultivation method appropriate for *Acanthamoeba*. Amoebae were identified using diagnostic features discernable by light microscopy.

A total of 283 water samples were processed and amoebae were noted in 80 of these. *Acanthamoeba* were found on 8 occasions (2.8%). The genera *Hartmannella* and *Vahlkampfia*, rarely involved in keratitis cases, were found in 3.5% and 2.8% of samples, respectively. A total of 19 different naked amoebae were recorded and amoebae (regardless of genus) were present in 19.4% of all samples.

Previous surveys in England and Korea have shown that acanthamoebae are found in 15 to 30% of tap water samples in the home and have been associated with corneal infection in contact lens wearers. The incidence of acanthamoebae infection in the USA (2.8%) has been found to be lower than that in the UK and it has been postulated that this is related to the lack of a storage water tank in the roof loft space. However, the level of treatment of municipal water is clearly not effective at killing amoebal cysts (or trophozoites) as evidenced by the high occurrence of amoebae (19.4%) in this study.

**Key words** | *Acanthamoeba*, contact lens, keratitis, tap water

### INTRODUCTION

It was demonstrated 15 years ago that tap water is an important factor in the etiology of *Acanthamoeba* keratitis infections (Seal *et al.* 1992). It is generally accepted that contact lens wearers who clean and rinse their lenses in tap water can introduce amoebae onto the lens surface, initiate colonization of the lens and provide the means for transferring an infective dose to the eye surface (Ledee *et al.* 1996; Kilvington *et al.* 2004). The use of tap water to clean lens storage cases can also result in the proliferation of amoebae within the case. In order for this to be possible, acanthamoebae must both be present in tap water and be able to withstand the rigors of water treatment plants,

primarily filtration and chlorination. Previous studies have shown the presence of acanthamoebae in domestic water supplies (Seal *et al.* 1992; Kilvington *et al.* 2004), however no such baseline study has ever been conducted in the U.S.

The present study attempted to find culturable acanthamoebae and other naked amoebae in domestic water supplies. The resistance of acanthamoebae cysts to chlorination was tested and the resistance of trophic amoebae, in general, was inferred from previous studies conducted on two species of *Vannella*. The present survey of domestic water was based on water samples collected throughout Broward, Dade and Palm Beach counties.

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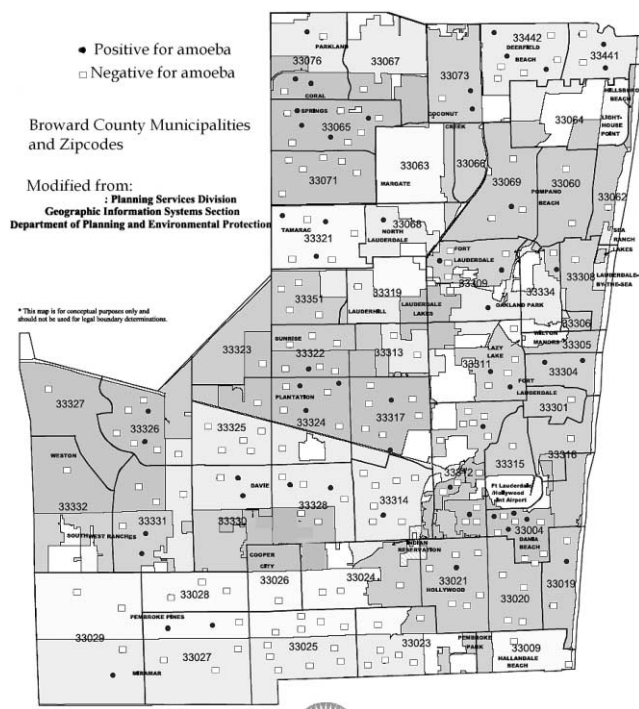
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The most common genotype implicated in *Acanthamoeba* keratitis infections is the T4 genotype. However, this genotype is also the most common type isolated from the environment, comprising some 72% of all acanthamoebae encountered (Booton *et al.* 2002). It follows that the prevalence of T4s in eye infections may simply reflect their abundance in nature rather than a predisposition to cause infection. Even so, the interest in genotypes and infection demanded that any isolated strains should be typed.

## METHODS AND MATERIALS

### Domestic water sampling for amoebae

Domestic water samples were collected from throughout Broward County over a two year period (Figure 1). Additional samples were taken from the neighboring south Florida counties, Dade and West Palm Beach. Although it would have been preferable to sample water directly from the inside



**Figure 1** | Map of Broward County indicating the city/zip code where water samples were taken. Solid circles indicate the water sample was positive for amoebae and empty boxes indicate the water sample was negative for amoebae. The samples from Dade and Palm Beach counties are not represented here; however, as in Broward County, no clear “hot spots” of amoebae were evident (Broward County Planning 2005).

surface of tap faucets (to collect some of the biofilm in the tap opening), most homes in the U.S. have mixer faucets that deliver water that is too warm for the survival of most amoebae. In Europe, some studies have sampled the storage water tanks common in the attic of homes. But again, such a system is not available in U.S. homes. Thus, in this study, the only consistent sampling site was the water storage tank (cistern tank) serving the toilet. This site has the advantage that it is constantly bathed in cold water supplied by the municipal water mains for that area. Since it is relatively undisturbed and rarely (if ever) cleaned, many tanks have a noticeable biofilm. It was this film that was sampled for amoebae.

Sample kits were assembled and distributed to students and colleagues willing to participate in this study. Each kit contained an alcohol wipe (Certified Safety Mfg., Kansas City, MO), a sterile cotton swab (BD Falcon, Sparks, MD), a sterile sample collection bag (Nasco, Fort Atkinson, WI) and a detailed set of instructions. In brief, the participant was instructed to clean his/her hands with an alcohol wipe before using the sterile cotton swab to thoroughly scrape a two-inch square area of the inner tank surface below the water line. This swab collected any surface-attached amoebae in the tank. The swab was placed in the sterile sample bag and sealed. Samples were returned to the laboratory (within 48 h) and processed while the swab tip was still moist. The swab tip was placed on a non-nutrient amoeba saline (NNAS) agar plate seeded with *E. coli*, an appropriate growth medium for acanthamoebae (Page 1988). After at least two weeks of incubation, the plates were rinsed with amoeba saline to dislodge amoebae and the washings were examined by light microscopy. If acanthamoebae were found, these amoebae were sub-cultured onto a fresh NNAS agar plate streaked with *E. coli*. Acanthamoeba were subsequently cloned by picking up a single amoeba off the agar plate with a sterile scalpel and transferring the cell to a new NNAS agar plate with *E. coli*. These clonal isolates were maintained in the laboratory until they were genotyped using methods previously described (Booton *et al.* 2002, 2005). Other naked amoebae recovered from domestic water (i.e. non-acanthamoebae) were photographed and identified (to genus where possible) using features discernable under phase contrast microscopy. These included cell size, mode of locomotion (eruptive, steady), and morphology of both the floating form and locomotive form. Identifications were made using keys by Page (1988) and Rogerson & Patterson (2000).

### Tolerance of acanthamoebae cysts to chlorine levels found in tapwater

Since the tap water survey showed that acanthamoebae were present in some domestic water supplies in south Florida, experiments were conducted to determine the tolerance of acanthamoebae cysts to two chlorine levels typically found in tap water. Five isolates of acanthamoebae with differing genotypes [DS(T4), S1(T11), #3(T5), P209(T4), and P120(T3)] were used to highlight possible correlations between genotype and tolerance to chlorine (Booton *et al.* 2002, 2004). Strains DS and #3 were from beach sand, strain S1 was from soil, strain P209 was from an AK patient in Hong Kong, and strain P120 was from tap water in Hong Kong (Booton *et al.* 2002, 2004). Household bleach (Ultra Clorox® Regular Bleach) was used to give concentrations of sodium hypochlorite up to 5.0 mg/L. Normally, levels found in tap water are much lower and rarely exceed 4.0 mg/L (7), however, periodically the County flushes the system with pulses up to 5.0 mg/L to inactivate biofilms in the system.

The test acanthamoebae were cultured on NNAS agar streaked with *E. coli*. Agar plates were left for at least three weeks to allow all cells to encyst. The cysts were washed off the plate using amoeba saline and the scraping action of a rubber policeman (BD Falcon, Sparks, MD). Cysts were transferred to a sterile culture tube (16 ml) containing amoeba saline (Page 1988) with the experimental concentration of chlorine (i.e. 3 mg/L or 5 mg/L). Experiments were replicated three times for each strain and level of chlorine. On each run, a control sample was processed (cysts in amoeba saline). Treatment time was 24 h in all cases at room temperature (ca. 23°C). After 24 h of treatment, the viability of the organisms was determined using a most probable number (MPN) dilution series (Blodgett 2003). The multiple tube technique used three sets of tubes containing amoeba saline (three replicates) which were inoculated with a ten fold difference in inoculum volume between each set: one set of three tubes were inoculated with 10 ml of treated organisms per tube, one set was inoculated with 1 ml treated organisms per tube, one set was inoculated with 0.1 ml treated organisms per tube and the last set of three tubes was inoculated with 0.01 ml of treated organisms per tube. Tubes were briefly vortexed (5 sec) before pipetting off volumes to ensure thorough mixing. To score for growth (from surviving

cells) in the respective dilutions, 20 µl aliquots were pipetted into 1 ml volumes of amoeba saline contained in six wells of a tissue culture plate. A small aliquot (2 µl) of Bacto-Casitone/Serum (BCS) medium (6) was added to stimulate the growth of attendant prey bacteria. This slight organic enrichment, and the subsequent development of a dense bacterial population in the wells, induced viable cysts to excyst and the emerging trophic amoebae to feed and reproduce. Each set was incubated at 21°C and examined for the presence of a growing amoeba population (derived from surviving cysts) after 3 and 5 d. The frequency of positive wells was used to generate a 'numeric' that was used with an MPN spreadsheet to determine the number of organisms surviving (Blodgett 2003). Percent survival was computed by comparing the MPN of the control after 24 h incubation to the MPN after the treatment time of 24 h.

MPN data for comparisons was weighted to account for the different starting concentrations of amoeba. Statistical analysis was done on the MPN data using single-factor ANOVA and the Tukey-Kramer procedure for determining differences in mean. Data was analyzed using the PHStat2 add-in for Microsoft Excel (Version 10, Prentice Hall, 2001).

## RESULTS

### Domestic water sampling for amoebae

A total of 283 domestic water samples were processed, and 55 were positive for amoeba (regardless of genus). In other words, 19.4% of all samples contained amoebae. A total of 19 different morphotypes of naked amoebae were isolated and identified (Table 1 & Figure 2). The most common morphotype, *Vexillifera* (Table 1, #1), was found in 7% of the water samples, twice as common as *Hartmannella* (Table 1, #2) which was seen in approximately 3% of water samples. *Acanthamoeba* (Table 1, #3) was the third most common morphotype found in water samples, occurring in 8 samples, or 2.8%. Of four acanthamoeba samples that were genotyped, three were T4's and one was a T5. Each morphotype shown in Figure 2 probably represents a different species.

As noted above, only 2.8% of all samples contained *Acanthamoeba*. Out of all the samples positive for amoeba, *Acanthamoeba* accounted for 14.6% of amoebae found in this survey. The genera *Hartmannella* and *Vahlkampfia*, also

**Table 1** | Percent occurrence of amoebae found in south Florida domestic water samples over 2-year period. Data from a total of 283 samples. Tentative identification of each morphotype is given with cell length

	Morphotype	Size ( $\mu\text{m}$ )	Instances	%
1	Vexillifera	10–20	20	7.07%
2	Hartmannella	23–42	10	3.53%
3	<i>Acanthamoeba</i>	25–40	8	2.83%
4	Vahlkampfia	22–34	8	2.83%
5	Vannella sp. 2	17–27	7	2.47%
6	Cochliopodium	42–51	4	1.41%
7	Vannella sp. 1	20–55	4	1.41%
8	Unidentified sp 2	6.8–10	3	1.06%
9	Limax sp 1	7.5	2	0.71%
10	Platyamoeba sp 1	42.5	2	0.71%
11	Unidentified sp 1	8.5–12	2	0.71%
12	Vannella sp 4	40–45	2	0.71%
13	Mayorella	25–34	2	0.71%
14	<i>Echinamoeba</i>	17	1	0.35%
15	<i>Parvamoeba</i>	6.8–8.5	1	0.35%
16	<i>Platyamoeba</i> sp 2	20–25	1	0.35%
17	<i>Saccamoeba</i>	12–20	1	0.35%
18	Unidentified sp 3	13.6	1	0.35%
19	Vannella sp 3	1–10	1	0.35%

rarely reported to cause amoebic keratitis (Hay et al. 1996), were found in 3.5% and 2.8% of all samples, respectively. Additionally, many samples (8%) contained unidentified cysts, ciliates, or flagellates (including amoeboflagellates such as *Cercomonas*). One sample contained nematode worms.

As shown in Figure 1 which summarizes all the sampling sites, there were no “hot spots” of amoebae across Broward County. This was also true for the limited sampling conducted in Dade and Palm Beach counties (sites not shown). It should be noted that although many samples were negative for amoebae, it does not rule out their presence at low levels. The protocols used were only appropriate for detecting amoebae in a relatively small area of biofilm (2 in<sup>2</sup>) within the domestic water supply of houses. Presence could be related to abundance in the film, time of year the sample was taken,

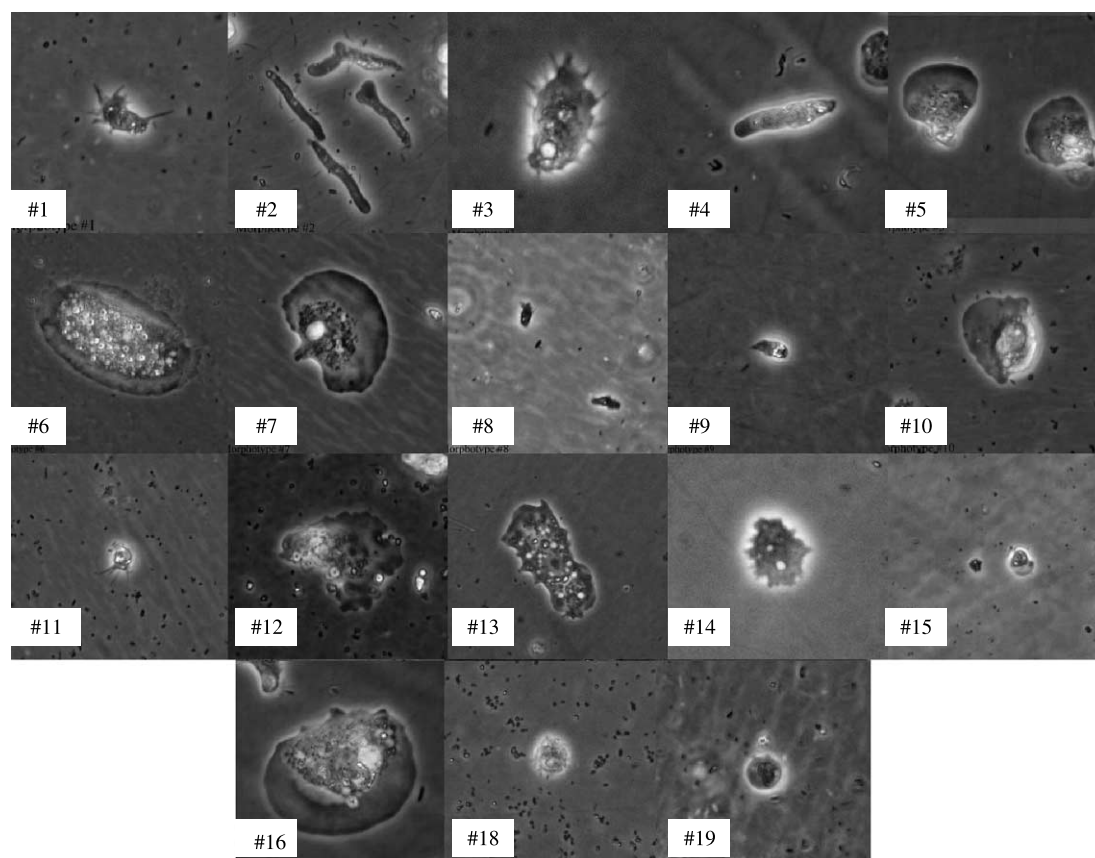
chlorine levels of the water, or thickness of the tank biofilm. None of these parameters were recorded for this study.

### Chlorine tolerance of cysts

Although no single *acanthamoeba* strain was killed (100%) by the experimental chlorine concentrations, single factor ANOVA and the Tukey-Kramer procedure indicated that there were significant differences between the different strains and their rates of survival. The cysts of the T4 genotype strains, DS and P209, were both quite resilient to chlorine showing 74% and 85% survival, respectively, in 3 mg/L chlorine and 49% and 89% survival, respectively in 5 mg/L chlorine. The tap water isolate P120 (genotype T3) was also resilient showing 95% survival at a chlorine concentration of 3 mg/L and 66% survival at a concentration of 5 mg/L. However, genotypes T11 and T5 (i.e. the environmental strains S1 and #3 respectively) were more susceptible to chlorine and many cells were killed at both the 3 mg/L and 5 mg/L concentrations. Strain S1 (genotype T11) had only 3% survival at 3 mg/L and 8% survival at the 5 mg/L concentration. Strain #3 (genotype T5) fared only slightly better with 24% of cysts surviving the 3 mg/L treatment and 16% surviving the 5 mg/L treatment. Single-factor ANOVA and Tukey Kramer tests were performed to determine if significant decreases in the experimental concentrations were significantly different from survival in the controls. Strains DS, S1, and #3 all showed significant decreases in cell density at both the 3 mg/L and 5 mg/L chlorine levels. Strain P120 did not show a significant decrease at 3 mg/L but did decrease significantly at 5 mg/L. The strain P209 did not show a significant decrease in survival rate at either 3 mg/L or 5 mg/L.

### DISCUSSION

Approximately 19.4% of all the water samples tested in south Florida (n = 283) were positive for naked amoebae. However, it should be noted that this is undoubtedly an underestimate of the true frequency of amoebae in domestic water. Only a small area of biofilm from the water system was sampled and the enumeration method used a culture method appropriate for *Acanthamoeba* isolation (the organism of concern). Not all amoebae can be cultured on the thin water



**Figure 2** | Morphotypes of different amoebae isolated on NNAS agar plates during domestic water sampling. Photomicrographs correspond to isolates given in Table 1. Morphotype 17 (*Saccamoeba*) is not illustrated.

film afforded by an agar plate. Thus, the data on morphotypes and frequency of occurrence are conservative. A wider range of culture methods and the processing of larger samples would have increased the values but perhaps not by much since growth on agar does satisfy the requirements of most small amoebae (the size of amoebae expected in tap water). The 19.4% occurrence of amoebae in domestic water samples was similar to levels reported by a different method used in the United Kingdom in 1992 (Seal *et al.* 1992) but lower than reported recently in the United Kingdom when up to 89% of cold tap water samples contained amoebae and 30% contained *Acanthamoeba* (Kilvington *et al.* 2004). This large difference is most likely due to the fact that in the UK the cold water is held in a large attic storage tank which supplies the home (Seal *et al.* 1992).

This difference in occurrence of acanthamoebae in UK tap water (11 times more abundant than reported in the present study) might explain the higher incidence of

*Acanthamoeba* keratitis incidence in the UK. This eye condition is 15 times more common in England and Wales than in the United States (Kilvington *et al.* 2004). Although incidences of acanthamoebae in tap water across the nation are not available, the low incidence of *Acanthamoeba* in the south Florida water supply supports this view. A larger, more comprehensive survey encompassing other states is needed. The current outbreak of *Acanthamoeba* keratitis in the Chicago land area (forty-four cases of *Acanthamoeba* keratitis in 2.5 years) warrants a closer look at that water supply in particular (Joslin 2006).

The survival of at least some acanthamoeba cysts in chlorine levels up to 5 mg/L shows that chlorination alone is not adequate for the elimination of amoeba cysts. It should be noted that this concentration is well in excess of levels routinely employed in water treatment systems (4 mg/L at the source with residual chlorine levels of around 0.2 mg/L in the distribution pipes). Thus it was not surprising that viable cysts

were recovered from the water samples. What is surprising is that 19.4% of samples processed yielded amoebae. This is far higher than would be expected from the recovery of a few rare cysts passing through the filters of the treatment plant. Rather, it suggests that there were substantial populations of active amoebae (trophs) in the biofilm sampled. This population may alternate between trophic and cyst forms depending on prevailing chlorination levels, but it does suggest that a growing population is maintained within the biofilm lining the distribution system. This notion is supported by a different study on the survival of trophs of a similar freshwater amoeba, *Vannella anglica* (Shoff 2006). Here, excysted amoebae tolerated up to 1 mg/L chlorine, a level often found downstream of treatment plants.

The presence of a growing population of acanthamoebae within the distribution system is clearly a cause for concern if tap water is a factor for the increased incidence of *Acanthamoeba* keratitis among contact lens wearers especially given the recent outbreak of *Acanthamoeba* keratitis in Chicago and several other US cities (Centers for Disease Control and Prevention 2007). It has been shown beyond doubt with genotyping that *A. griffini* (T3) can colonize bathroom tap water, the contact lens (CL) storage case and adhere to the CL to cause severe keratitis (Ledee et al. 1996). Contact lens wearers should be told to avoid storing their lenses in tap water and to use a multi-purpose solution instead. However, as published by one of the authors 14 years ago, *Acanthamoeba* keratitis is an infection that is 'here to stay' (Seal 1994).

The presence of viable amoebic cysts in one fifth of all samples tested has other implications. Clearly, this shows that the present method of water treatment is inadequate for either the removal of small cysts (ca 15 µm and smaller) or their inactivation through disinfection. It is likely that cysts of obligate pathogens such as *Cryptosporidium*, *Giardia*, and *Entamoeba* would also survive the treatment process. These parasitic protozoa are of greater concern since the 'cyst' stage is transferred to the host by ingestion. Monitoring water supplies for these pathogens is not straightforward and relies on immuno-detection methods. Monitoring for surrogate protozoa such as naked amoebae cysts might prove to be a useful technique in the future if an improved standard is demanded.

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