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Acanthamoeba in the Domestic Water Supply of Huntington, West Virginia, U.S.A.

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Summary. The aim of this study was to determine the prevalence of *Acanthamoeba* in the domestic water supply in Huntington, West Virginia (U.S.A.) and the factors that may contribute to their presence or absence. One hundred sixty-two one liter tap water samples were collected over eight months. Amoebae in the samples (cysts or trophozoites) were harvested by passively filtering onto 5 µm pore size filters and enriching for amoebae on non-nutrient amoeba saline agar plates seeded with *Escherichia coli* for cultivation. Thirteen percent of all samples were positive for amoebae and 9.3% were positive for the amoeba of interest, *Acanthamoeba*. Chlorine levels were determined for samples at the time of collection, yielding a mean level of 1.56 mg l^{-1} chlorine in the distribution system ca. 8 kilometers from the water treatment plant. Cysts and trophozoites of *Acanthamoeba* clonal isolates were found to tolerate up to 50 mg l^{-1} and 4 mg l^{-1} chlorine respectively. This study showed that *Acanthamoeba* were present in the domestic water supply in Huntington, WV and although no attempt was made to count cells in liter samples, their frequency of occurrence (9.3%) and failure to be present in all replicates, suggests they were present at background levels of perhaps a few cells per five liters. This is only the second U.S. study to consider amoebae in tap water and is unique since the source water was river water. *Acanthamoeba* trophozoites and cysts were able to withstand levels of chlorine higher than those typically found in tap water suggesting they may be present in either form in the distribution system. *Acanthamoeba* are opportunistic pathogens capable of causing eye infections and their presence in tap water is a potential risk factor for susceptible individuals, particularly contact lens wearers who may use tap water to clean lenses and storage cases.

Key words: Amoebic Keratitis, amoebae, tap water, *Acanthamoeba*, chlorine tolerance.

INTRODUCTION

According to Page (1988), acanthamoebae may be the most commonly encountered genus of naked amoeba in freshwater and soil habitats. Even a few small samples are likely to yield isolates of *Acanthamoeba*, testament to its prevalence and ubiquity in nature. On rare occasions, some strains of these free-living, bactiverous amoebae, can become opportunistically pathogenic and invade human tissue (Marciano-Cabral and Cabral 2003). The most frequently encountered infection is Amoebic Keratitis (AK), a sight-threatening invasion of the cornea (Seal *et al.* 1998). The factors that promote invasion of the eye are unknown but contact lens wearers are at most risk possibly because the lens surface, if contaminated,

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can transfer an infective dose of amoebae to the corneal surface (Kilvington *et al.* 2004). Additive, or alternate, factors include the molecular genotype of the amoeba (Ledee *et al*. 2009), corneal trauma (Sun *et al*. 2006) or ineffective lens cleaning methods (Booton *et al.* 2002). With regard to the latter, the use of tap water contaminated with amoebae (Shoff *et al.* 2008) or the inability of current lens cleaning formulations to kill or inactivate acanthamoebae may be important (Shoff *et al.* 2007).

The prevalence of *Acanthamoeba* eye infections within contact lens wearers is uncertain although Lam *et al.* (2002) claim 0.33 cases per 10,000 lens wearers per year. Recently, interest in acanthamoebae has grown following an increase in AK cases in Illinois, U.S.A. This outbreak prompted the Center for Disease Control (Atlanta, 2007) to initiate a national investigation to look for risk factors associated with this increase. Despite this interest, we still have no clear information on what factors lead these amoebae to invade the eye however their possible occurrence in domestic water supplies and the (non-recommended) practice of using tap water for cleaning storage cases (Houang *et al.* 2001) does underscore the need to monitor water supplies. Previous studies have shown acanthamoebae in domestic water supplies (Seal *et al.* 1992, Kilvington *et al.* 2004, Jeong and Yu 2005) but only one study has looked at U.S. supplies. Shoff *et al.* (2008) reported on the prevalence of *Acanthamoeba* in south Florida domestic water and found them present in 8 out of 283 water samples.

It is likely that the source of the domestic water and the method of treatment (commonly filtration, ozonation and/or chlorination) have bearing on densities of amoebae and other protists within water supplies in different geographic regions. In Florida, drinking water is taken from a shallow aquifer and this may explain the relatively rare occurrence of acanthamoebae, although other species of amoebae were found and overall 19.4% of samples tested were positive for naked amoebae (19 species) (Shoff *et al*. 2008). Although not examined in previous studies, it is likely that water from deep aquifers would contain very few, if any, *Acanthamoeba*. On the other hand, in Huntington, WV, water is collected directly from the Ohio River leading to the possibility that source water may be rich in *Acanthamoeba,* especially after rain events when soil washes into the river.

Since *Acanthamoeba* have been shown to resist levels of chlorination typically used in water treatment (Shoff *et al*. 2008) the present study was undertaken to determine whether culturable amoebae are present in the water distribution system of Huntington.

MATERIALS AND METHODS

Water sampling

Over an eight month study period, tap water samples were collected from four homes within a five mile radius of Huntington's (WV, U.S.A.) water treatment plant. Additional comparative samples were collected from neighboring Hurricane, WV a town of some 6,000 people (2006 census data) 28 miles East of Huntington. This community is not on the Ohio River and is served by a different treatment plant. Volunteer collectors were instructed to let the cold water run for 60 seconds prior to collecting water samples. On each sampling event, at least three one-liter samples were collected from the water faucet into separate sterile media bottles. Samples were returned to the laboratory within one hour, and concentrated by passively (no vacuum) filtering the water through a 5 µm pore size Nuclepore filter (Fisher Scientific, Pittsburg, PA.). Gentle filtration concentrated any amoebae (both trophozoites and cysts) in the sample onto the filter surface that retained a thin layer of water. Moist filters were transferred to non-nutrient amoeba saline (NNAS, Page 1988) agar plates streaked with the prey bacterium *E. coli*. Plates were incubated for seven days at room temperature (ca. 23°C), washed with amoeba saline and the suspension observed by phase contrast light microscopy (× 400) for the presence of amoebae. These were identified to genus using diagnostic features discernible by light microscopy (Page 1988). When *Acanthamoeba*, the amoeba of interest, were present stock clonal cultures were prepared. Individual cells that had migrated over the agar surface well away from the population were noted using a dissecting microscope. A small block of agar containing a single cell was dissected from the plate using a sterile scalpel. The block was placed face down on a fresh NNAS plate seeded with *E. coli* and the clonal population was allowed to grow. Stock cultures were transferred every 8 weeks.

Chlorine tolerance of *Acanthamoeba* cysts and trophozoites

Cysts or trophozoites of four clonal *Acanthamoeba* isolates from tapwater were tested for their tolerance to chlorine. Methods used were similar to those described by Shoff *et al.* (2007). *Acanthamoeba* were inoculated onto NNAS plates streaked with *E. coli*. Exponentially growing cultures were used as the source of trophozoites. After about 2 weeks, the cultures had exhausted all prey and formed cysts. Experiments on tolerance of cysts used cultures between 2 and 4 weeks in age to ensure that only mature cysts were tested (Kilvington and Anger 2001). For trophozoite tolerance, blocks of agar (ca. 1.0 × 1.0 cm) containing ca. 50 amoebae were dissected from exponentially growing cultures and placed in the wells of a 24-well tissue culture plate made of uncoated polystyrene (Corning Incorporated, Corning, NY). Wells contained a single block with amoebae facing up. The blocks were covered with amoeba saline containing sodium hypochlorite at final test concentrations of 2 and 4 mg l^{-1} chlorine. Solutions were prepared fresh for each trial from chlorox® bleach (52,500 mg l^{-1} sodium hypochlorite). For each concentration, between 6 and 36 replicate blocks were set up. Preliminary experiments using fewer replicates (n = 3) were used to zero in on these threshold concentrations that defined the effective concentration. Trophozoites were incubated at room temperature (ca. 23°C) for 24 h. Controls were run in parallel using blocks in amoeba

saline. Following incubation, blocks were gently rinsed in sterile amoeba saline and placed amoeba-side down on an *E. coli* streak (NNAS agar plates). Plates were wrapped in parafilm, incubated at room temperature and observed under a dissecting microscope (× 60 with transmitted light) after four days. Plates were scored for the presence or absence of amoebae; surviving trophozoites formed dense populations after incubation. In the case of cysts, procedures were identical excepted that stock cultures were 2 to 4 weeks old and concentrations of chlorine were higher because of the tolerance of mature cysts. In this case, effective concentrations (determined by preliminary experiments) spanned 25 and 50 mg l^{-1} chlorine.

Molecular identification of tapwater isolates

Four tap water isolate clones morphologically identified as *Acanthameoba* were grown on NNAS plates seeded with *E. coli* as described above, washed from plates using amoeba saline, and DNA isolated using Qiagen's DNeasy Blood & Tissue Kit (Cat. No. 69504: Valencia, CA) according to the manufacturer's protocol. For molecular identification of isolates (i.e. genotyping), PCR amplification was carried out using *Acanthamoeba*-specific primers and full procedures as detailed in Schroeder *et al.* (2001). Sequences were deposited in GenBank with accession numbers for isolates M1, M9, M10 and M12 designated as GU586221, GU586222, GU586223, and GU586224, respectively.

Chlorine testing of tapwater

On each sampling occasion, a water sample (10 ml) was collected for chlorine analysis. These analyses were made immediately upon collection using a portable Photometer standardized to read total chlorine (Orion research Inc., Beverley, MA).

Statistical analysis

Z-tests of proportions were run using SigmaStat version 3.5. Trendline for figure one was generated using Excel 2007.

RESULTS

A total of 162 one-liter tapwater samples were processed over the course of this study from the main study area (Huntington WV). Samples were collected on 12 sampling events in Huntington. Although four different taps were sampled (all within a one mile radius) there were no discernible differences regarding the presence of naked amoebae. Overall, 21 samples were positive for amoebae (Table 1) representing 13.0% of all samples. The genus *Acanthamoeba* was the predominant type (9.3% of samples were positive for this amoeba). For comparison, a few samples (12 one-liter) were collected from a much smaller, regional water treatment plant in Hurricane, WV. Here, chlorine levels were much lower averaging just 0.035 mg l^{-1} chlorine (relative to the mean level of 1.56 mg l^{-1} in the Huntington water samples; with 0.86 mg l^{-1} the lowest and 2.31 mg l^{-1} the highest level observed during this study period). This suggests this factor may have affected the prevalence of amoebae in the samples from Hurricane, WV, with 41.7% yielding naked amoebae compared to 13% from Huntington samples (z value = 2.263, p = 0.024). And all of the positive samples from Hurricane contained the genus of interest, *Acanthamoeba.*

It is important to note that no attempt was made to count the actual number of amoebae present in a sample. The data merely reflect the presence or absence of amoebae and do not address whether one-liter samples contained one or many amoebae. However, the large number of negative scores where no amoebae were found (87% of samples in Huntington) suggests that amoebae were not numerically important in samples. It is also relevant to note that in all cases multiple bottles were sampled at each site (between three and 12 one-liter bottles) and on no occasion were all bottles positive for amoebae. If amoebae were periodically numerically important in the distribution system, all replicates would have yielded amoebae. Even so, when more than one morphotype (presumed species) of amoeba was present, this suggests that amoebae were numerically more abundant. While chlorine levels in the water were not the only factor that could control the presence or absence of amoebae, it did appear to be an important variable. The low chlorine levels in Hurricane water led to more positive results and to the maximum diversity found in a sample, namely 3 morphotypes that were morphologically distinct at the light microscope level. The relationship between chlorine and types of amoebae is given in Fig. 1. Here, many samples failed to yield amoebae (regardless of chlorine levels, triangular data points), however, the positive samples (diamonds) do correlate with chlorine levels. Extrapolation of the data would suggest that no morphotypes would be found at around 2.6 mg l^{-1} chlorine (chronic exposure).

Table 1. Occurrence of amoebae in domestic water samples from Huntington, WV and Hurricane, WV.

Huntington, WV	No. samples	% of total samples
Samples positive for amoebae	21	13.0 (21/162)
Samples positive for *Acanthamoeba* sp.	15	9.3 (15/162)
Samples positive for amoebae	5	41.7 (5/12)
Samples positive for *Acanthamoeba* sp.	5	41.7 (5/12)

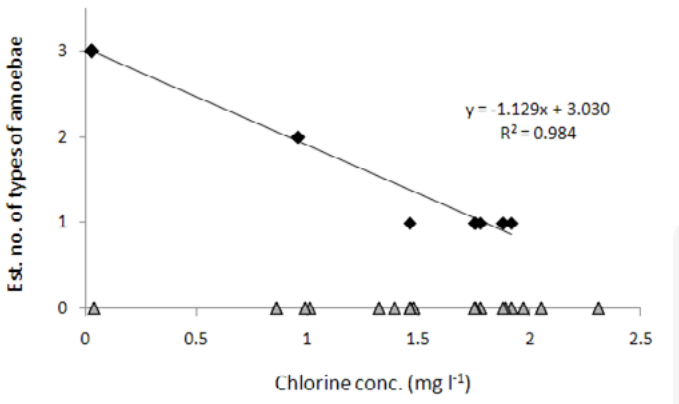

Fig. 1. Regression of estimated number of types of amoebae cultured from sampling events against chlorine concentrations of water samples. Samples positive for FLA = (diamonds) and negative for FLA = (triangles).

Surprisingly, only 3 different morphotypes (presumed genera) were present over the course of the sampling. These were identified as *Acanthamoeba*, *Vannella* and an unidentified limax amoeba. This low diversity was partly due to the culture conditions employed. Only some amoebae can multiply in the thin water film afforded by an agar plate (NNAS/*E. coli*). Even so, many more amoebae can be successfully cultured on agar than reflected in this study. The Culture Collection of Algae and Protozoa (CCAP, U.K.) houses some 55 genera of amoebae and almost half can be grown on agar formulations (A. Rogerson, pers. comm.).

Acanthamoeba was the most frequently encountered amoeba in this study (in 20 samples out of 174, 11.5% [from both locations]). The identification of this genus is easy because of the distinctive morphology of trophozoites (spiny pseudopodia) and cysts (stellate inner wall) (Page 1988). Even so, to confirm their identity, four random isolates were cloned and subjected to PCR analysis using *Acanthamoeba*-specific primers according to established methods (Schroeder *et al.* 2001). The approximately 450 bp amplimer obtained for each of these four clones was gel purified and sequenced. Sequences were subjected to a BLAST search against all available sequences in GenBank (Zheng *et al*. 2000). All four sequences yielded matches to *Acanthamoeba* sp. sequences of the T4 genotype (96–98% sequence identities).

Four *Acanthamoeba* tap water clonal isolates were tested for their tolerance to levels of chlorine typically found in domestic water supplies (Table 2). The maximum allowable level set by the U.S. Environmental Protection Agency (EPA) is 4 mg l^{-1} at source, and levels detected in samples taken throughout this study were all below that level. Trophozoites (Table 2a) were treated for 24 hours in either 2 or 4 mg l^{-1} chlorine. As shown in Table 2a, reduced survivability was observed at 4 mg l^{-1} compared to 2 mg l^{-1}, but these levels never resulted in 100% killing for any of the four isolates tested. Significantly less survival was observed at 4 mg l^{-1} compared to 2 mg l^{-1} (determined using a z-test of two proportions; $z = 3.946$; $p = < 0.001$). Cysts from all four isolates survived treatment with 25 mg l^{-1} chlorine (Table 2b) and cysts from two of the isolates survived treatment at 50 mg l^{-1} chlorine. Lower levels of chlorine were initially tested (< 25 mg l^{-1}) and cysts were unaffected at these lower levels (data not shown). Results are reported here starting with 25 mg l^{-1} as a drop-off in survival was observed beginning at this level of treatment (Table 2b). Significantly less survival was observed at 50 mg l^{-1} chlorine treatment compared to 25

Table 2. Percent survival of trophozoites and cysts of *Acanthamoeba* sp. tapwater isolates when treated with different levels of chlorine.

a. Trophozoites

	Chlorine conc.			
Clone #	2 mg l^{-1}		4 mg l^{-1}	
M1	80.00	(n = 30)	66.67	(n = 36)
M9	75.00	(n = 24)	40.00	(n = 30)
M10	100.00	(n = 12)	44.44	(n = 18)
M12	100.00	(n = 12)	72.22	(n = 18)

b. Cysts

	Chlorine conc.			
Clone #	25 mg l^{-1}		50 mg l^{-1}	
M1	100.00	(n = 6)	0.00	(n = 6)
M9	58.33	(n = 12)	66.67	(n = 12)
M10	100.00	(n = 18)	0.00	(n = 18)
M12	83.33	(n = 6)	100.00	(n = 6)

c. Summary of all tap water isolates

	Chlorine conc.			
	2 mg l^{-1}	4 mg l^{-1}	25 mg l^{-1}	50 mg l^{-1}
Trophozoites	84.62	55.88	n/d	n/d
Cysts	n/d	n/d	85.71	33.33

mg l^{-1} (determined using a z-test of two proportions; z = 4.668; p = < 0.001). As shown here, cysts were only affected at chlorine levels much higher than would be encountered in the domestic water supply. Control blocks of cysts or trophozoites treated with amoeba saline (no chlorine) all showed growth on NNAS with *E. coli*.

DISCUSSION

Poor hygiene practices among contact lens wearers seem to be a major factor in the etiology of *Acanthamoeba*-induced Amoebic Keratitis (Seal *et al.* 1999). One such practice is rinsing or storing lenses in tap water leading to contamination of lens cases (Seal *et al.* 1992). Up to 15.7% of lens storage cases in Korea were contaminated with acanthamoebae (Kong *et al.* 2002). Of course this supposes that these amoebae are present in domestic water supplies and the few studies that have considered this do attest to their presence (Seal *et al.* 1992, Kilvington *et al.* 2004, Jeong and Yu 2005, Lorenzo-Morales *et al.* 2005, Shoff *et al.* 2008, Boost *et al.* 2008). The only previous study from the U.S. was a survey of domestic water in south Florida (Shoff *et al.* 2008). Naked amoebae were present in 55 out of 283 samples and acanthamoebae were present in 8 samples (2.8%). The other studies from England, Spain, Hong Kong and Korea found higher incidences of acanthamoebae in home tap water. In England, Kilvington *et al.* (2004) found acanthamoebae in 26.9% of tap water samples, in Spain 59.5% of tap water samples yielded acanthamoebae (Lorenzo-Morales *et al.* 2005), 10% in Hong Kong (Boost *et al.* 2008), and in Korea 7.7% of domestic tap water samples were positive (Jeong and Yu 2005). Although none of these studies are directly comparable, because the source water and treatments at the water plants varied as did the method of sampling, they do illustrate the potential of tap water to harbor viable acanthamoebae either as trophozoites or cysts. And besides the potential pathogenic properties of *Acanthamoeba* spp., their ability to serve as hosts for pathogenic bacteria further underscores the importance of monitoring their presence in domestic water supplies (Xuan *et al*. 2007, Laskowski-Arce and Orth 2008, Thomas *et al*. 2009).

The present study is the second baseline study from the U.S. to report on the prevalence of *Acanthamoeba* in tap water. Unlike the Florida study, where water was taken from a shallow aquifer, in West Virginia the water was taken from the Ohio River where more amoebae would be expected. By considering just the samples from the city of Huntington, 9.3% were positive for acanthamoebae and 13% for free-living amoebae. The identity of the genus *Acanthamoeba* was confirmed for 4 representative clonal cultures using molecular genotyping. All were T4 genotypes, the commonest environmental *Acanthamoeba* and the one most frequently isolated from AK patients (Seal *et al.* 2003, Booton *et al*. 2004).

Another variable between geographic locations is the degree of chlorination of the domestic water supply. In the U.S., the maximum permissible level at source is 4 mg l^{-1} chlorine (U.S. Environmental Protection Agency mandate). In Florida, the levels were much lower in the distribution pipes, around 0.2 mg l^{-1} chlorine, and noticable biofilms were evident on the inside of the water storage tank (cistern tank) that served the toilet (Shoff *et al.* 2008). In Shoff's study the film was scraped and used to inoculate plates to isolate amoebae. The low chlorine levels and evident slime layer may explain the high diversity of amoebae found, 19 species in the 19.4% of positive samples. In the present study, only 3 morphotypes (presumed species) were found and highest diversity was found at the lowest chlorine levels. Indeed, water treatment in Huntington relies on heavier doses of chlorine averaging 1.56 mg l^{-1} chlorine. It is also worth noting that when the cistern tank was observed there was no noticeable biofilm coating the inside. Of course these studies are not directly comparable since one sampled the biofilm and the other sampled tap water directly, however, they do point to the importance of chlorine as a factor in determining diversity and probably the abundance of naked amoebae in tap water.

The tolerance of 4 isolates of acanthamoebae to chlorine levels was tested and 2 mg l^{-1} had little or no effect on strains while 4 mg l^{-1} killed or inactivated cells on approximately half the replicate agar blocks (Table 2). It is important to note that a 50% survival does not mean that half the cells were killed. Rather it means that half the replicate agar blocks containing 50 cells failed to grow. In other words, it is likely that some of the surviving blocks had partial kills but enough viable cells survived to produce a positive grow-out. Clearly, survival is impacted around the 2–4 mg l^{-1} chlorine level which matches the data presented in Fig. 1 where a mean background level of 2.6 mg l^{-1} would prevent the growth of trophozoites. Cysts were very resistant to chlorination and were unaffected until levels reached 25

and 50 mg l^{-1} chlorine, well in excess of any concentrations expected in a water treatment plant. These results were similar to those reported by Shoff *et al.* (2008) and suggest that cysts are unaffected by treatment and most trophic amoebae would survive in less than 2 mg l^{-1} chlorine although there could be sublethal effects.

Chlorine susceptibility raises the issue about possible growth of acanthamoebae in the distribution system. In the Florida study, the very high diversity and the obvious biofilm prompted the authors to speculate that there were active populations of growing amoebae in the biofilms lining the distribution system (Shoff *et al.* 2008). On the other hand, the higher chlorine levels in Huntington and the corresponding lack of a bacterial biofilm suggest that acanthamoebae were transient in the domestic supply, either passing through the treatment plant as trophozoites or as cysts.

The source of amoebae in this study was ultimately the Ohio River water, however, given the numerical importance of amoebae in sediments and soils, many more morphotypes were expected, although it must be noted that culture grow-out conditions (i.e. on agar surface) only allows for the isolation of a subset of the heterotrophic protists. Even so, the three genera of amoebae encountered over the course of the study were far less than expected. Moreover, no other types of protozoa (ciliates and heterotrophic flagellates) were found. Since sand filters employed in the treatment plant would not remove the diversity of small protists inhabiting the river, it is assumed that most protozoa are susceptible to the effects of chlorine at levels used although there is very little comparable data available on this topic. In the present study, cysts of the *Vannella* found in tap water were also very resistant to chlorine (survived in 50 mg l^{-1} chlorine, data not shown). M. Shoff (pers. comm.) has shown the flagellate *Chilomonas* and the freshwater amoebae *Vannella platypodia* show significant decreases in water at 1.0 and 0.5 mg l^{-1} chlorine, respectively. Until more data is available on chlorine tolerance in protozoa, it is assumed that the three types isolated had unusual resistance. Certainly, some strains of acanthamoebae can survive in multipurpose contact lens cleaning solutions as both trophozoites and cysts (Shoff *et al.* 2007, Johnston *et al.* 2009), an indication of their tolerance to chemical disinfectants (Turner *et al.* 2000).

Related to the topic of source, it was assumed that the number of amoebae (in this case at least one culturable cell per 1 l sample) would increase on days following heavy rain events when the source river water was filled with runoff from the soil. But this was not the case and there was no consistent pattern between frequency of positive isolations and timing of rain events (data not shown). This opens the possibility that the treatment plant is acting as a source of these resistant protozoa and that there is a constant flushing of cells from populations growing in the sand filters. Amoebae are well adapted to inhabiting the bacterial rich crevices on sand grains and perhaps there are sizable populations of acanthamoebae growing in the filters analogous to the populations found on gravel beds in sewage treatment plants. This notion is not without support. A recent European study, also a river fed water treatment plant, reported that sand filters were colonized and may occasionally release FLA into filtered water (Thomas *et al.* 2008). Most importantly, they reported that *Acanthamoeba* and *Naegleria*-related amoebae were recovered from sand filtration units. Viewing a sand filter as a source of these amoebae in a water treatment plant is highly speculative but does warrant further study since acanthamoebae are persistent opportunistic pathogens.

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