Res. Microbiol. 152 (2001) 753–760

S0923-2508(01)01256-6/FLA

Biofilms augment the number of free-living amoebae in dental unit waterlines

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Received 12 March 2001; accepted 18 April 2001

Abstract – Freshwater amoebae are ubiquitous. Some species can cause infections in humans while others can ingest and protect opportunistic bacteria. Although the presence of free-living amoebae in various water sources has been reported, few studies have looked at their concentration, which may be clinically relevant, especially if they are present in healthcare devices. A simple technique was used to detect, observe, and evaluate the concentration of free-living amoebae in dental unit and tap water samples. Fifty-three water samples were collected from 35 dental units (air/water syringes) and 18 water taps. The technique was based on the ability of waterborne bacteria to create a biofilm and serve as substratum for the development of amoebae naturally present in the water samples. Laboratory-grown freshwater biofilms support the proliferation of a wide variety of free-living amoebae. All the dental unit water samples tested contained amoebae at concentrations up to 330/mL, or more than 300 times the concentration in tap water from the same source. *Hartmanella, Vanella*, and *Vahlkampfia* spp. were the most frequently encountered. *Naegleria* and *Acanthamoeba* spp. were also present in 40% of the samples. Four of the samples collected from dental units, but none from water taps, contained amoebae able to proliferate at 44 °C. Biofilms that form inside some dental instruments can considerably increase the concentration of free-living amoebae, some of which are potential human pathogens. © 2001 Éditions scientifiques et médicales Elsevier SAS

free-living amoebae / dental units / biofilm

1. Introduction

Free-living amoebae and cysts are present in most aqueous environments including lakes and ponds, drinking water, thermal discharges, eyewash stations, dental unit waterlines, and hemodialysis units [12, 14, 23, 31, 35, 44]. They have been found airborne in their very resistant cystic form [20, 34], which may explain their presence in the noses and throats of some individuals [21, 33]. Some species of free-living amoebae such as *Naegleria* and *Acanthamoeba* are known human pathogens [33, 45]. *Naegleria fowleri* has been linked to lethal primary amoebic meningoencephalitis, an acute fulminating disease [13, 49], while *Acanthamoebae* spp. can induce painful, vision-threatening infections of the cornea in contact lens wearers, especially if the lenses are colonized by bacterial biofilms [24, 39, 40]. *Acanthamoeba* spp. can also cause granulomatic amoebic encephalitis as well as brain, pulmonary, and kidney infections [24, 41, 44]. Direct contact with or exposure to dust or aerosols containing free-living amoebae or cysts may cause infections [1, 7]. The ability of amoebae to induce infections is likely dependent on virulence factors, heat tolerance [19], and host susceptibility. However, the infectious dose is unknown. More recently, it has been suggested that amoebae and cysts can act as “Trojan horses” for pathogens like *Legionella pneumophila* and nontuberculous mycobacteria [7, 10, 40, 43].

High levels of opportunistic pathogens in water used for high-speed dental drills and air/water syringes and hemodialysis care units have been reported [5, 11, 47]. The water column inside the small lumen moves in the center of the tubing leaving a thin layer of liquid virtually undisturbed against the walls. This physical state creates propitious conditions for water microflora to establish a successful and tenacious biofilm. The large (6:1) area-to-volume ratio of small waterlines gives biofilm plenty of surfaces on which they can spread and a relatively small volume of liquid to fill with shedding daughter cells.

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Concentrations of *Pseudomonas aeruginosa*, nontuberculous mycobacteria, and *Legionella pneumophila* are several times higher in dental unit waterlines than in regular tap water [2, 4, 9]. The formation of waterborne biofilms, which act as reservoirs for opportunistic pathogens [4, 6, 47], may in large part be responsible for these observations. Freshwater amoebae, which have been reported to be present in dental unit water samples [25, 26] and hemodialysis care units [12] may even be responsible for the high concentrations of *Legionella* and mycobacteria reported [2, 8, 38]. Amoebae have also been observed grazing on biofilms that build up inside small-bore waterlines [6].

The detection of protozoa and especially free-living amoebae in water samples is usually time-consuming and is based on nonnutrient agar seeded with specific live or heat-killed bacteria. In addition, the samples sometimes need to be concentrated by centrifugation or filtration with an inherent loss in recovery [32]. Although such techniques provide reliable results as to the frequency of positive samples, actual enumeration of amoebae requires additional steps [16]. This may explain why few publications give the concentration of amoebae in water samples despite the fact that this information may have important implications in terms of health risk assessment.

Amoebae feed on bacteria, and it has been postulated that biofilms may favour the growth and expansion of the populations of bacterial predators naturally present at low levels in drinking water. We used a simple method based on the natural ecology of freshwater biofilms to detect, observe, and evaluate the concentration of amoebae in water samples.

2. Materials and methods

2.1. Water samples and cultivation of amoebae

Fifty-three water samples were collected from 35 dental units (air/water syringes) and 18 water taps at the Université de Montréal. All the samples were collected directly in 50-mL sterile plastic tubes (Sarstedt, Germany). Six of the 35 dental unit samples were collected after a two-minute flush, which is the time recommended for reducing the bacterial load in water. A 9-mL volume of each sample was inoculated into polystyrene 25-cm^2 culture flasks (Corning, NY) containing 1 mL of 10 × R2A medium (0.5 g starch, 0.5 g yeast extract, 0.5 g tryptic peptone, 0.5 g glucose, 0.3 g K_2HPO_4, 0.05 g $MgSO_4$, 0.25 g succinate, and 0.5 g casamino acids in 50 mL distilled water). The remainder was used for enumeration assays as follows. Fourfold dilutions of the water samples (up to 1:256) were carried out in sterile distilled water. Four wells of 24-well polystyrene tissue culture plates (Corning, NY) were seeded with 900 µL of each dilution. Each sample was carried out in duplicate giving 8 wells for each dilution. One hundred microliters of 10 × R2A was then added to each well. The plates and flasks were left undisturbed at room temperature for 72 h in the dark. The presence of a biofilm was confirmed when an adherent layer of bacteria covering the bottom of the wells or flasks was observed using an inverted microscope at a magnification of 400 ×. The wells and flasks were then rinsed three times with sterile distilled water to remove nonadherent bacteria and the R2A medium was replaced with sterile distilled water. The incubation was continued at room temperature for two weeks. The plates were examined daily for the presence and development of amoebae using an inverted microscope. The optical density of the supernatant was maintained below 0.100 (600 nm) at all times by replacing the content of the culture vessels with sterile distilled water. An optical density greater than 0.150 slowed the proliferation of amoebae or caused encystment and interfered with visual observation of the amoebae using the inverted microscope.

2.2. Heat tolerance

Ten-milliliter water samples were collected in 15-mL screw cap test tubes. To allow the growth of potentially pathogenic amoebae, which would otherwise have been masked by other amoebae, the test tubes were incubated at 44 °C for 24 h. The contents were then transferred to 25-cm^2 culture flasks. To allow the formation of a biofilm, 1 mL of R2A (10 ×) medium was added to the flasks, which were then incubated at 37 °C for 3 to 5 days until a biofilm had formed. The culture medium was then withdrawn and replaced with 10 mL of sterile distilled water. The incubation was continued at 37 °C until a proliferation of amoebae (indicated by the formation of microcolonies) was observed. The temperature was then raised to 44 °C and the cultures examined at 1, 3, 6, and 24 h for changes in amoebae locomotion and morphology, or encystment. If the trophozoites were still active and proliferating after 24 h, the flasks were left at 44 °C. If encystment occurred, the temperatures was adjusted to 37 °C and the cultures were observed for 24 h for de-encystment. The transfer to 44 °C was repeated twice more. Active locomotion and proliferation at 44 °C was considered to be indicative of thermotolerance.

2.3. Identification

The morphological criteria of Page [29, 30] were used to identify the amoebae. Size measurements were performed using a scaled graticule. Amoebae movement and locomotion were also recorded using an inverted microscope equipped with a JVC color video camera. Some samples were examined by electron microscopy after gently scraping the flasks with a cell scraper to recover the amoebae. After a 10-min centrifugation at 500 g, the pellets were embedded in 5% agar and processed for electron microscopy as described below.

Selected flasks were directly stained with crystal violet for the observations of the amoebae in relation to biofilms. Supernatant was discarded and the flasks were rinsed 3 times with distilled water to remove nonadherent cells. Two mL of Gram crystal violet was added and left in contact with the biofilm layer for 5 min. Flasks were then rinsed 3 times with tap water and observed with the inverted microscope.

2.4. Enumeration of amoebae

We used Fisher's method [17] for negative cultures to evaluate the concentration of amoebae in water samples incubated in the 24-well microplates. Briefly, we calculated x as the mean fertile level with $x = X/n$, where X is the total number of fertile (positive) wells (considering all the wells of the two 24-well plates, without taking the dilution into consideration) and n is the number of wells seeded for a given dilution. Thus, $log\lambda = xloga - K$, where λ is the number of organisms per well at the highest concentration, a is the dilution factor, and K is a constant taken from a mathematical table (table $VIII_2$ in reference [17]). Each well was observed daily for at least two weeks at a magnification of 400 ×. A well was considered positive if at least one cyst/amoeba was detected. Statistical analyses were performed using the Mann-Whitney U test.

2.5. Electron microscopy

Agar-embedded pellets and sections of dental unit waterline and the associated biofilms were prefixed for 30 min in 0.2% glutaraldehyde and 0.15% ruthenium red (RR) in 0.1 M cacodylate buffer at pH 7.4, followed by a 2-h fixation in 1% glutaraldehyde with 0.05% RR in the same buffer. After several buffer washes, the samples were postfixed in 1% osmium tetroxide and 0.5% RR for 1 h. The samples were dehydrated in graded concentrations of ethanol, embedded in LR white resin (Marivac, Halifax, NS, Canada), and polymerized at 58 °C. Thin sections of the tubing were cut with a diamond knife on a Reichert Ultracut E ultramicrotome, recovered on formvar and carbon-coated nickel or gold grids, and examined with a JEOL JEM-1200 Ex TEM operated at an accelerating voltage of 60 kV.

3. Results

3.1. Culture and observations

In vitro freshwater biofilms were used to increase the concentration of amoebae in water samples. These natural biofilms served as the feeder layer for the development of the amoebae. All 53 water samples contained amoebae at varying concentrations (see section 3.4). After 3 to 4 days of incubation, dense bacterial growth and biofilm formation were observed as indicated by the presence of an adherent layer and scattered microcolonies and streamers on the bottom of the flasks and the wells of the plates containing R2A medium. *Bodo* spp. flagellates as well as ciliates were also detected in some samples. Conspicuous proliferation of amoebae was observed 48 to 72 h after replacing the R2A medium with sterile distilled water (*figure 1C*). Both trophozoites and cysts were clearly visible using an inverted microscope at 400 × magnification. Clear circular 2 to 20 mm zones (plaques) on the biofilm could be seen with the naked eye in some samples within 72 h (*figure 1A*). These plaques corresponded to a centrifugal expanding ring of bacteria-laden amoebae feeding on the biofilm (*figure 1B*). The number of plaques, and their diameters, increased over the following four days until the plaques coalesced and the biofilm was depleted. Amoebae generating these plaques were identified as *Naegleria* spp. After 10 days, over 90% of the amoebae had encysted. Using

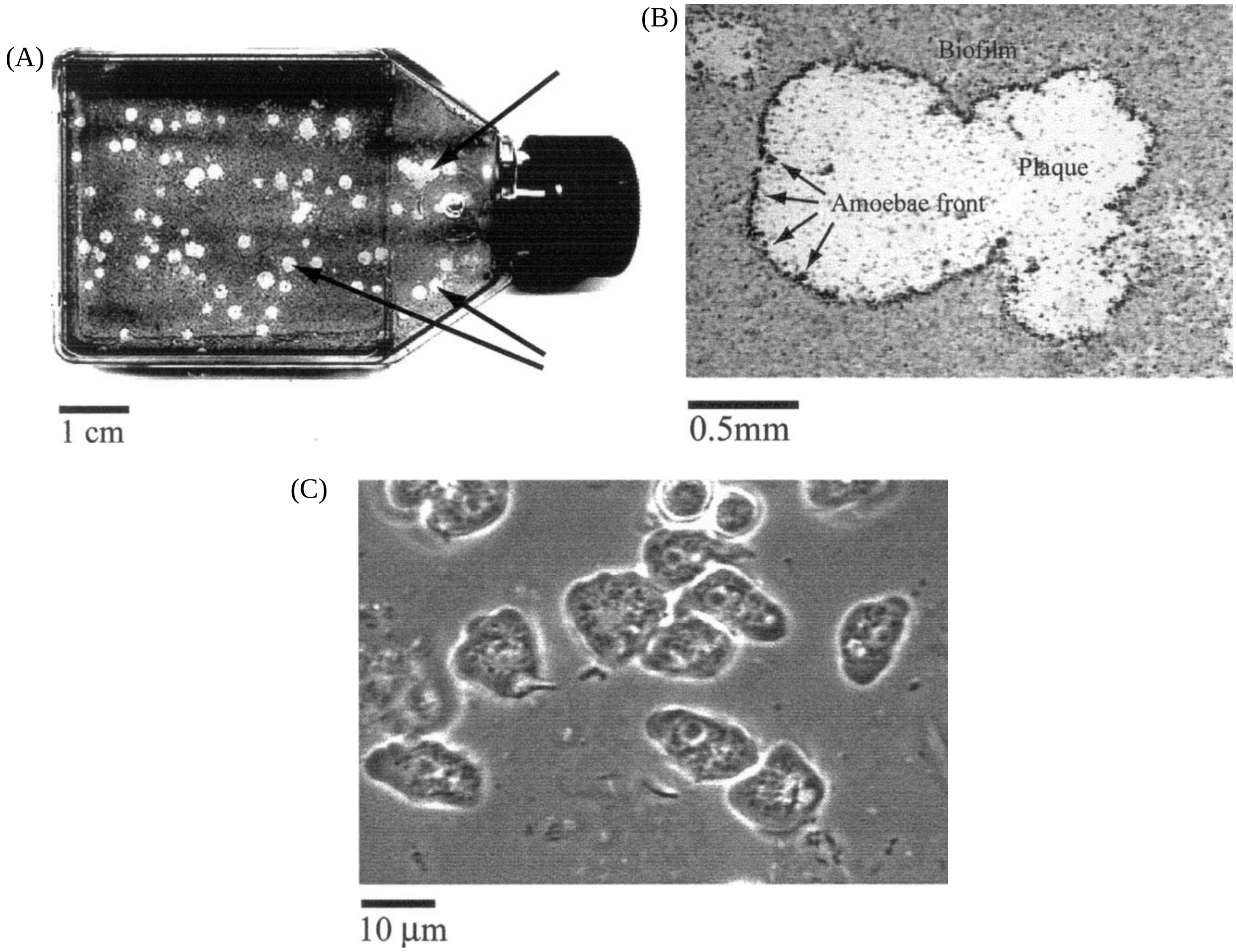

Figure 1. (A) Clear circular 2–20-mm zones (arrows) in the biofilms could be seen with the naked eye in some samples within 72 h. Crystal violet staining. (B) These zones (plaques) corresponded to a centrifugal expanding front of amoebae feeding on the biofilm. (C) The proliferation of a variety of free-living amoebae was evident 5 to 6 days after inoculation.

our culture technique, we recorded an average of 5×10^5 cysts/25-mL flask. The high turbidity of the overlaying supernatant inhibited the proliferation of the amoebae but generally could be overcome by extensive rinsing with sterile water to reduce the free-floating microbial biomass.

3.2. Electron microscopy

Sections of nine dental unit waterlines were examined using electron microscopy. Trophozoites were occasionally observed in biofilm samples taken directly from waterlines. Some were observed in the process of phagocytosis at the surface of the biofilm, with bacteria trapped in vacuoles (*figure 2A*). Spherical cysts were more common and were located at the surface of the biofilm or embedded in the matrix (*figure 2B*). When grown in culture, amoebae could be observed actively ingesting bacteria (*figure 2C*).

3.3. Identification and thermotolerance

No differences were noted between water samples collected from dental unit waterlines and water taps with respect to amoebae species. *Vanella platypodia*, *Vanella miroides*, *Hartmanella vermiformis*, and

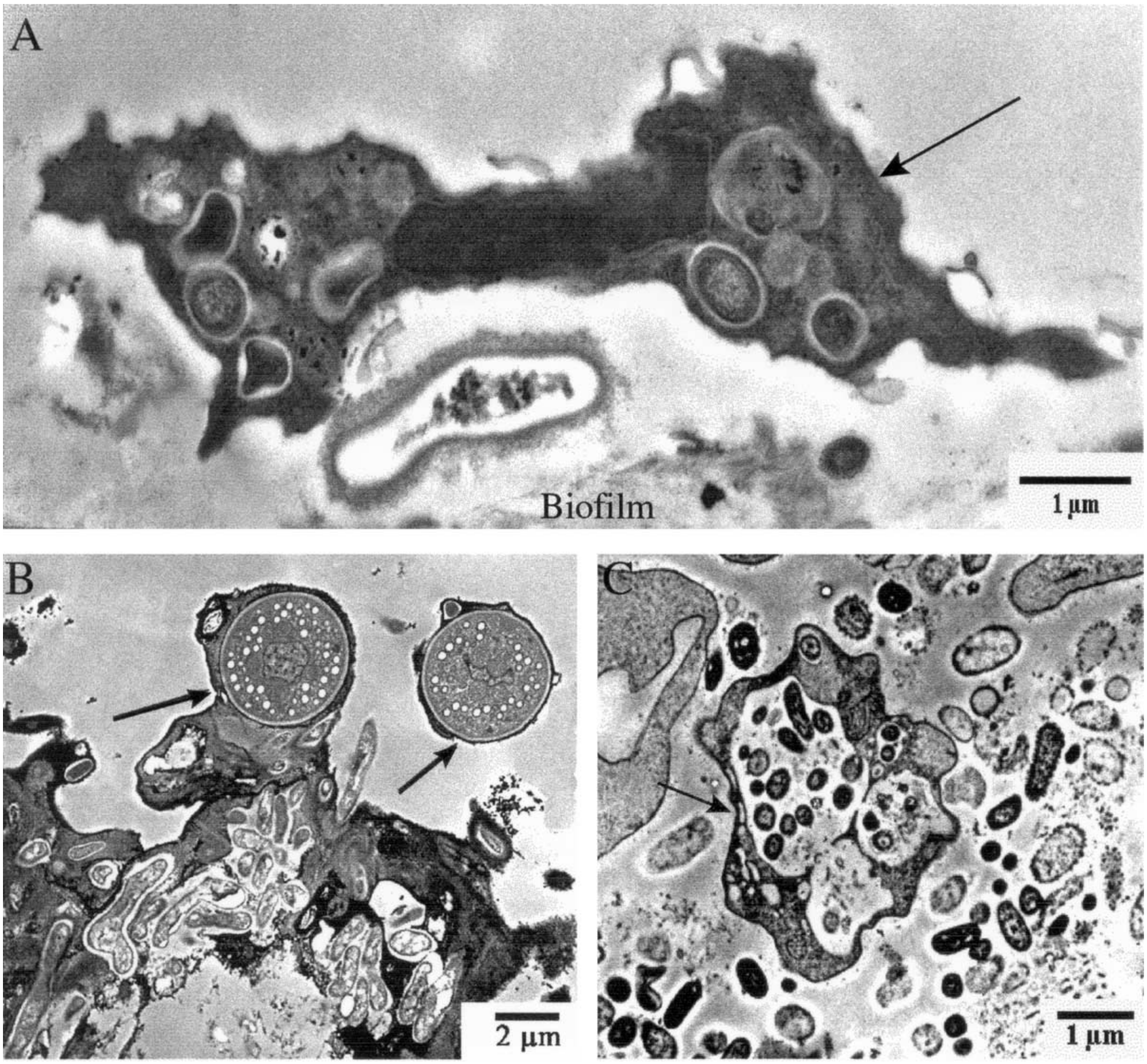

Figure 2. Micrographs of a biofilm from inside a dental unit waterline. (A) Amoebae (arrow) in the process of phagocytosis are occasionally observed at the surface of the biofilm. (B) Cysts (arrows) are common features and these structures are sometimes embedded in the biofilm matrix. (C) In culture medium, amoebae actively ingest bacteria, explaining the gradual destruction of the biofilm structure.

Vahlkampfia spp. were most frequently encountered. *Filamoeba nolandi* was isolated from a single water sample. *Acanthamoeba* and *Naegleria* spp. were detected in 40% of all our samples. Bitrichous *Bodo* spp. were also observed. All 53 samples contained amoebae that grew at room temperature (21 °C). Four of the 35 dental unit water samples (11.4%) contained *Vahlkampfia* spp. that grew and proliferated at 44 °C. No tap water samples yielded growth at 44 °C.

3.4. Enumeration of amoebae

Final results were recorded 15 days after the enrichment procedure had been initiated. All the water samples contained amoebae. Water taken from dental units before flushing had much larger amoebae populations than tap water. The median number of amoebae in dental unit and tap water samples was 83.22/mL (332.89–4.46) and 1.12/mL (5.30–0.94) respectively (*figure 3*). This difference is highly signifi-

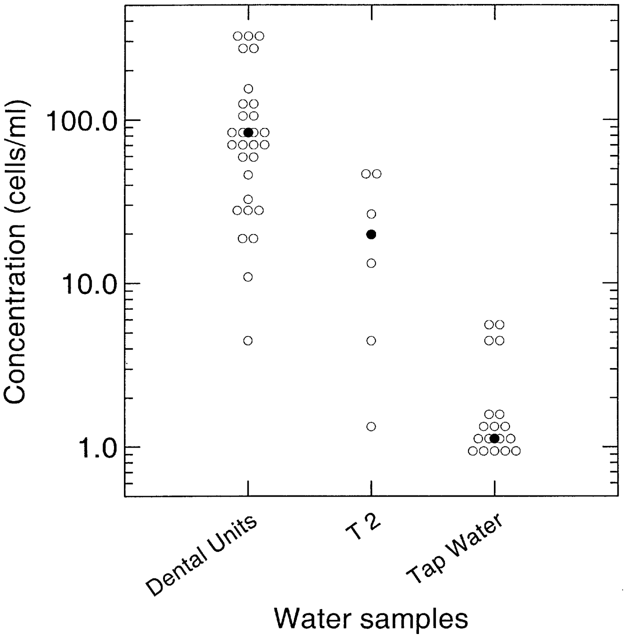

Figure 3. Concentration of free-living amoebae in tap water and in dental unit water samples before and after a 2-min flush (T2). Black dots represent the median for each sample group.

cant ($p < 0.0001$). Flushing the dental unit waterlines for 2 min reduced the amoebae concentration by 66% (median $= 19.71$; $p = 0.006$).

4. Discussion

It is well known that dental unit waterlines are heavily populated with bacteria. The lumen of the small-bore tubes are colonized by a tenacious freshwater biofilm that acts as a reservoir for opportunistic pathogens like *Pseudomonas aeruginosa, Legionella pneumophila,* and nontuberculous mycobacteria, among others. As a consequence, bacterial counts up to several million per mL can be reached in water samples (review in [4]). The same findings have been reported for haemodialysis care units [11, 22]. The high concentrations of legionellae and mycobacteria in dental unit water samples may, in part, be due to the presence of amoebae in the microenvironment created by the tubing. Different amoebae species may carry *Legionella* spp. and mycobacteria as endosymbionts [27, 28]. Biofilm formation may also favor the proliferation of amoebae, thus creating an ecological loop.

Using our technique, we were able to detect amoebae in all water samples tested using small volumes of water. This technique is a modification of older methods such as placing coverslips in liquid media to stimulate amoebae development. One drawback of the coverslip method is that only amoebae or cysts that adhere resist elimination during the rinsing steps. However, the culture vessels we used are treated for optimal cell adherence by the manufacturer. We made no attempt to control or select particular bacterial species, assuming that naturally occurring species would be the best for selecting freshwater amoebae.

The concentration of free-living amoebae is up to 300 times higher in dental unit water samples than in tap water even though the water source is the same. This may be explained by the combination of the biofilm itself, the large (6:1) area-to-volume ratio of the small-bore waterlines, which gives the biofilm a large surface to colonize, and the relatively small volume of liquid to fill with shedding bacteria and amoebae [3]. Amoebae feed on bacteria and waterborne biofilms are probably good sources of prey, as indicated by electron microscopy micrographs. Even though trophozoites can occasionally be seen by direct observation – both optical and electron microscopy – amoebae are mainly present as cysts in waterlines. The high concentrations of bacteria in waterlines may somehow cause encystment. This assumption stems from our observation that, when cultured, the amoebae stopped proliferating and encysted when planktonic bacterial concentrations rose above 10^5/mL. Such concentrations can easily be reached in dental unit waterlines. The inhibitory effect of high concentrations of bacteria on amoebae has also been reported by Wang and Ahearn [48].

No differences were noted between dental unit waterlines and water taps with respect to amoebae species diversity. Although *Hartmanella* and *Vanella* spp. were most frequently encountered, *Acanthamoebae* and *Naegleria* spp were also isolated. This is in agreement with the work reported by Michel et al. [25, 26]. However, there is no mention of the concentration of amoebae in the water samples, a factor that may be of clinical significance. For instance, *Acanthamoeba* keratitis may arise when a biofilm forms in contact lenses case allowing pathogenic amoebae to proliferate and reach an infectious level [39]. It has been reported that the formation of circular plaques by amoebae in culture is a characteristic feature of *Naegleria* spp. and could be used as a means to segregate between pathogenic from nonpathogenic *Naegleria* spp. Differentiation is based on the size of the plaques and the sharpness of the leading edge [15]. Although we found that circular plaques were associated with *Naegleria* spp. in our samples, no attempt was made to further characterize those plaques.

It has been shown that *Hartmanella* and *Acanthamoebae* spp. not only can ingest *Legionella* spp. but also can stimulate their proliferation and increase their virulence [45]. This may also be true for nontuberculous mycobacteria [42]. Sanden et al. [37] found that preincubation of water samples for several days increased the recovery of *Legionella* spp. and that this was related to the proliferation of amoebae in the samples. This phenomenon may also exist inside dental unit waterlines.

The ability of amoebae to grow at temperatures above 44 °C is regarded as an indication of pathogenic potential for *Nagleria* and *Acanthamoeba* spp. However, thermotolerant amoebae are mainly associated with hot water systems or warm ponds. Four out of 35 of our samples grew at 44 °C. These results were similar to those of Rohr et al. [36], who reported that 17% of isolates from moist areas (presumably at ambient temperature) were able to grow at 44 °C. The clinical implications of our findings are unknown. However, since water-cooled high-speed drills and air/water syringes generate bioaerosols [18], mucosal contact or inhalation of amoebae or cysts by dental workers and patients cannot be ruled out. Regular flushing of the lines before health care procedures, as recommended by some dental associations [3], may improve water quality by lowering bacterial and amoebae counts.

Acknowledgements

We would like to thank Pierre Rompré for the statistical analyses, Gaston Lambert for the excellent photographs and Gene Bourgeau for editorial assistance. This work was supported by the sterilizer monitoring service of our laboratory.

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