Title: Risk assessment for the spread of *Serratia marcescens* within dental-unit waterline systems using *Vermamoeba vermiformis*

Authors: Sham Lal[a], Sim K. Singhrao[a]*, Undine E.M. Achilles-Day[b], L. H. Glyn Morton[a], Mark Pearce[a], StJohn Crean[a]

[a]Oral & Dental Sciences Research Group,

School of Medicine and Dentistry,

University of Central Lancashire,

Preston, PR1 2HE.

[b]Culture Collection of Algae and Protozoa

Scottish Association for Marine Science (SAMS)

Scottish Marine Institute

Argyll, PA37 1QA

Scotland.

Running Title: *Serratia marcescens* from DUWLs

*Corresponding author:

Dr S. K. Singhrao, Tel: +44 (0) 1772 895137; Fax: +44 (0) 1772 892965

E-mail: SKSinghrao@uclan.ac.uk

Details of co-authors

- Sham Lal
  E-mail: slal1@uclan.ac.uk
  Tel: 07438566203

- Undine E.M. Achilles-Day
  E-mail: Undine.Achilles-day@sams.ac.uk
  Tel: 01631 559000

- Prof. L. H. Glyn Morton
  E-mail: LHGMorton@uclan.ac.uk
  Tel: 01772 894373

- Mark Pearce
  E-mail: oliver.pearce@tiscali.co.uk
  Tel: 01282 773490

- Prof. StJohn Crean
  E-mail: screan@uclan.ac.uk
  Tel: 01772 895902

Conflict of interest disclosure statement

All named authors declare that there is no conflict of interest.

Title: Risk assessment for the spread of *Serratia marcescens* within dental-unit waterline systems using *Vermamoeba vermiformis*

Abstract:

Vermamoeba vermiformis is associated with the biofilm ecology of dental-unit waterlines (DUWLs). This study investigated whether *V. vermiformis* is able to act as a vector for potentially pathogenic bacteria and so aid their dispersal within DUWL systems. Clinical dental water was initially examined for *Legionella* species by inoculating it onto *Legionella* selective-medium plates. The molecular identity/profile of the glassy colonies obtained indicated none of these isolates were *Legionella* species. During this work bacterial colonies were identified as a non-pigmented *Serratia marcescens*. As the water was from a clinical DUWL which had been treated with Alpron™ this prompted the question as to whether *S. marcescens* had developed resistance to the biocide. Exposure to Alpron™ indicated that this dental biocide was effective, under laboratory conditions, against *S. marcescens* at up to 1×10^8 colony forming units/millilitre (cfu/ml). *V. vermiformis* was cultured for eight weeks on cells of *S. marcescens* and *Escherichia coli*. Subsequent electron microscopy showed that *V. vermiformis* grew equally well on *S. marcescens* and *E. coli* (p = 0.0001). Failure to detect the presence of *S. marcescens* within the encysted amoebae suggests that *V. vermiformis* is unlikely to act as a vector supporting the growth of this newly isolated, nosocomial bacterium.

Key words: *Vermamoeba vermiformis,* non-pigmented *Serratia marcescens*, dental-unit waterlines

Introduction:

Dental-unit waterlines (DUWLs) consist of fine narrow bore tubing that extends to approximately six metres in length [9]. The DUWL is an essential component of the modern day dental treatment unit that supplies water to cool the dental drill-tip and avoids causing heat-related damage to the soft, pulpal nerve tissues of healthy teeth [24, 48, 51]. It has long been recognised that the DUWL readily harbours a microbial biofilm [11, 29] and that the discharged water can contain very high planktonic bacterial and protozoan loads, which could lead to the exposure of patients and health care workers to an increased risk of infection [26]. The Centers for Disease Control and Prevention (CDC) in the USA, made recommendations to the manufacturers of the dental units that they should have a separate reservoir, typically a container of about a litre, attached to the dental unit from which tap water, deionised water and/or distilled water can be fed to the drill to cool it. Such external reservoirs are also better suited for the introduction of biocides. This measure has been introduced by the CDC in dentistry for delivery of safe treatment water [22], although, there are working dental units that are fed directly from municipal water. Where biocides are used to control the contamination levels of the DUWL output water, their use is questioned for a number of reasons; it is considered that as a result of their activity biocides may encourage biofilm formation, introduce bacterial resistance and furthermore, are limited in their ability to control the diverse range of microbes associated with the DUWL heterogeneous biofilm community [13, 14].

It has now become apparent that there is a problem, common to all forms of man-made tubing, which attracts microbes from the water that flows through them and this is the phenomenon of biofilm formation. Even a newly commissioned DUWL with anti-microbial coating on its internal surfaces, which is maintained according to the recommended daily decontamination protocols [12], will rapidly develop a microbial biofilm which is then sustained throughout the entire life of this clinical device. Health care providers accept that bacteria will always persist in the dental treatment water, but the emphasis now lies on reducing the microbial loading of the discharged water to meet the CDC recommend level of ≤ 500 colony forming units (cfu) of aerobic mesophilic heterotrophic bacteria/millilitre, for infection control in dental health care system (22). Although, the American Dental Association (ADA) has set its own heterotrophic bacterial load of ≤ 200 cfu/ml for water delivered from DUWLs [2], ADA also endorses the CDC recommendation that patient treatment water should be the same quality as the Environmental Protection Agency (EPA) standard of ≤ 500 cfu/ml [2, 22]. The recommended standards for dentistry set by the Department of Health, in the UK, are of ≤ 200 cfu/ml [1, 3, 22]. These reports highlight a risk to patients and stress the importance of maintaining and delivering clean water during dental treatment, hence, it is important to assess the risk factor of microbial propagation by amoebae.

A variety of human pathogenic bacteria including *Legionella* have been isolated from DUWLs, by various researchers [5, 36, 42, 50, 58]. Furthermore, non-tuberculosis *Mycobacterium* species [41, 47], *Klebsiella pneumoniae* [37, 57], *P. aeruginosa* [16, 26] and *S. marcescens* [30, 44, 57] have also been identified. Despite tight controls to make sure the treatment water is safe, an elderly patient died from legionellosis following dental treatment in which *L. pneumophila* serogroup I was identified using molecular profiling from isolates taken both from the patient and from the clinical environment of the dental practice where treatment was performed, confirming the source of infection [43]. Circumstantial evidence also surrounds the death of a dental practitioner due to the same cause in the early nineties [5]. A report by Oppenheim et al. [35] describes a near-miss of a clinical infection on a larger scale with *L. pneumophila* exposure from aerosols generated from dental drills within a teaching institute.

Although the majority of waterborne bacteria pose no risk of infection, guarding against the risk to health of opportunistic nosocomial pathogens including, *Legionella*, non-tuberculosis *Mycobacterium* species, *K. pneumoniae*, *P. aeruginosa* and *S. marcescens*, to an ever growing list of people with immunocompromised status remains a cause for concern.

Amoebae feed on mixed communities of bacteria within biofilms including, pathogenic bacteria such as species of *Legionella*, *Mycobacterium*, *P. aeruginosa*, *Vibrio cholerae*, *Helicobacter pylori* [6, 12, 45, 46, 59]. Once inside the amoebal cell, some bacteria will survive or escape the adverse conditions presented by digestive vacuoles, but can also find sanctuary from unfavourable environmental conditions and can multiply [25]. Furthermore, bacteria such as *S. aureus* may be ingested, but released undigested and intact [39]. This implies that amoebae can transport certain species of nosocomial bacteria within their ecological niches [56].

Free living amoebae, *Legionella*, *Mycobacterium* and yeast species including *Candida*, found to reside within DUWLs are sourced from the tap water shared by domestic users and the healthcare providers alike [10, 25]. In the context of healthcare, the greatest challenge to overcome is when pathogenic nosocomial bacteria use free living amoebae to support their growth [7, 8, 31, 37, 43]. Lawsuits can be brought against the dental practitioner if causal links are confirmed between an infection and the use of dental treatment water [8].

S. marcescens is known to be a nosocomial pathogen which can acquire antimicrobial resistance [27]. This bacterium can cause a variety of infections in the susceptible host including septicaemia, meningitis, endocarditis and blindness [17, 20, 52]. Previous work from our laboratory on grazing habits of *V. vermiformis* [14] indicated that small sized bacteria were favoured as a food source. The small size of *S. marcescens* makes it an ideal target for protozoa to graze on. Furthermore, it is known that protozoa can support bacterial growth in

aquatic ecosystems and a prior study by Cateau et al. [12] implied that *P. aeruginosa* can be propagated through *V. vermiformis* and this could also be true for *S. marcescens*.

This study therefore tested the hypothesis that by grazing on *P. aeruginosa*, and other potential human pathogens *V. vermiformis*, (CCAP 1534/16) could promote the growth of these bacteria within the DUWL aquatic ecosystem. Thus highlighting the risk factor associated with pathogenic bacteria commonly found in DUWL systems grazing alongside other free living amoebae.

Materials and Methods

Sample collection

The proposed study was a service evaluation. Approval from the relevant NHS authorities concerning research governance (R & D North West) was obtained (proposal No 310) and general dental practices in the North West of UK, were subsequently, approached and asked for their willingness to participate in the study. In all, 31 practices consented and all of them conformed to a biocide water treatment recommended by the manufacturer of their dental chairs. The consenting practices were visited on mutually agreed days between 10.00 am and 12.00 pm and DUWL water (100 ml) from the air/water syringe was sampled. A number code was assigned to each sample from which output water was taken (DUWL 1 to 31) for traceability. Samples were transported in a cool box at 4°C to the laboratory at the Biomedical Research Facility, at our academic institute for further analysis.

Isolation of bacteria from clinical DUWL water including testing for *Legionella* species

In addition to performing conventional viable cell counts of aerobic mesophilic bacteria on R2A agar plates, three (100 μl) replicate samples of the water were also inoculated onto commercially prepared GVPC medium (Glycine-Vancomycin-Polymyxin-Cycloheximide) plates (Fisher Scientific) to test for the presence of *Legionella* spp. The GVPC plates were incubated at 30°C in a humid environment for up to seven days.

Maintenance of newly isolated individual bacterial colonies

Discrete bacterial glassy colonies growing on GVPC plates (suggestive of possible *Legionella* species), designated DUWL 9, 10 and 21 were picked and sub-cultured onto fresh GVPC medium and incubated at 30°C as described previously. Following incubation, colonies were tested for their Gram reaction and molecular

identity. Subsequently, they were inoculated onto R2A medium and nutrient agar at temperatures between 15 and 37°C, for maintenance and to assess the incidence of pigmentation.

Molecular identification of bacterial colonies

Following sub-culture, some colonies grew well under laboratory conditions on GVPC plates. Genomic DNA was isolated from 10 different colonies from each of the three DUWLs chosen for likely *Legionella* species of bacteria (DUWL 9, 10 and 21) and analysed for molecular identity using the method described previously by Dillon et al. [14].

The effect of a dental biocide on *S. marcescens* isolated from clinical DUWL 9 output water

The efficacy of Alpron™ was tested on pure laboratory cultures of *S. marcescens*, (the bacterium isolated from DUWL 9) together with cultures of *P. aeruginosa* (NCTC 10662) as control organisms.

Culture maintenance

E. coli (XL blue), *P. aeruginosa* (NCTC 10662), and *S. marcescens* from DUWL 9 were maintained by aseptically transferring cultures onto R2A plates and were incubated at 30°C for 2 days.

Bacterial liquid cultures

The antimicrobial testing was undertaken using 18h culture in nutrient broth at 30°C in a shaker set at 200 rpm.

Dilution profiles/regimes

The log phase bacterial cultures were centrifuged using a Sigma 3-16PK bench top centrifuge at 4,000 g for 20 min at 4˚C (Sigma-Aldrich Ltd., Dorset, UK). The resulting pellets were washed and re-suspended three times in 10 ml of sterile Ringer's solution made from ¼ strength Ringer's solution tablets (Lab M Ltd., Bury, UK) and the final suspension was held on ice until needed.

Assessment of resistance to Alpron™ dental biocide

A 1 in 10 dilution of commercial Alpron™ is recommended by the manufacturer as the daily working dilution for use within the device/system. The active ingredients in Alpron™ are sodium hypochlorite and a mixture of citric acid anhydrite with non-ionogenic tensides and dye.

The dilution in this study was prepared within ¼ strength Ringer's solution (Lab M Ltd., UK) containing 100µl of each bacterial suspension (final concentration of bacteria at 1×10^8 cfu/ml) for laboratory use. The controls

consisted of 100µl of bacterial suspension added to Ringer's solution (900µl). After approximately 12 h contact time with the biocide at room temperature, each suspension was serially diluted and inoculated on R2A agar plates using the Miles & Misra [32] method. The plates were incubated at 30°C for up to 7 days and examined after 24 h, 2 days and 7 days using a colony counter.

Phase-contrast and differential interference contrast microscopy

V. vermiformis (CCAP 1534/16) isolated and maintained in the laboratory as described by Dillon et al. [13] were placed onto a glass slide containing sterile isotonic saline solution and examined directly under a Zeiss Axio Imager A2 microscope. Images were taken using a Zeiss AxioCam HRc digital camera. For the image acquisition, phase-contrast and differential interference contrast microscopy methods were employed.

Maintenance of *V. vermiformis*

At the start of the experiment *V. vermiformis* was maintained on *E. coli* following the procedure described previously [13].

Preparation of fresh, live bacterial feed

Strains of *E. coli*, *P. aeruginosa*, and the newly isolated *S. marcescens* from DUWL 9 were maintained on R2A plates at 30°C for 3 days. On the 3rd day, each bacterium was taken and placed as food lines onto R2A plates to feed *V. vermiformis* at 22°C for seven days as described previously [13].

V. vermiformis feeding on *E. coli*, *P. aeruginosa*, and *S. marcescens*

The density of cells growing on a 1cm^2 plug of agar was calculated by detaching amoebae and suspending them in 2 ml of 1x PBS. A 10 µl aliquot was taken and used to count cells. This was carried out using a standard cell counter (haemocytometer) [14]. Plugs of agar with equivalent numbers of *V. vermiformis* on their respective bacterial feeds were taken weekly for up to 8 weeks. This procedure was carried out in triplicates. The plates were incubated at 22°C for 5 days. Following incubation, the plates were examined for growth and the area onto which amoeba had migrated over the R2A agar plates was measured to calculate the total number of cells/unit area as described previously [14].

Transmission electron microscopy (TEM)

Specimen preparation

In order to establish whether *V. vermiformis* was a carrier for *P. aeruginosa* and the newly isolated *S. marcescens* from DUWLs 9, three plates with amoebae grown on their respective feed (*P. aeruginosa* or *S. marcescens*) were incubated at 22˚C for 5 days. Amoebae were subsequently transferred from the plate by gentle re-suspension in a small volume of neutral pH phosphate buffered saline (PBS). The cells were collected into a Falcon™ 15 ml conical centrifuge tube (BD Biosciences) and pelleted by centrifugation at 1500 rpm for 30 min with further washings in between in PBS prior to fixation in neutral buffered glutaraldehyde (2.5%) for 3h at 4°C. Following fixation, the pellets were processed for embedding in Araldite as described previously [14].

Sectioning, examination and image capture

Thin sections of each specimen were cut using glass knives at 80-100 nm thickness using the Leica Ultracut E microtome and examined under an electron microscope as described elsewhere [14].

Statistical analysis

Where appropriate, data are presented as the mean $\pm$ SD (N = 3), tested for normality and equal variances, and analysed by one-way ANOVA (Minitab 16 statistical software and the IBM SPSS statistics20). Differences were considered significant at $p \leq 0.05$.

Results

Phase-contrast and differential interference contrast microscopy

The *V. vermiformis* (CCAP 1534/16) under phase contrast and differential interference contrast microscopy demonstrated their limax (trophozoite) morphology and round cysts (Fig. 1).

Molecular identification of bacteria from GVPC plates

Molecular sequencing of the 16s rDNA gene and subsequent Nucleotide BLAST search (ebi.ac.uk) identified the newly isolated bacterium from DUWL 9 as a non-pigmented *Serratia marcescens*. *Phyllobacterium myrsinacearum* from DUWL 10 and *Mycobacterium llatzerense* from DUWL 21; all with sequence similarities of 98-100% encompassing >200 bases [14]. *S. marcescens* gave a 98% nucleotide similarity, whilst *P.*

myrsinacearum gave 100% and *Mycobacterium llatzerense* gave 99% nucleotide similarity. No *Legionella* species were identified from any of the colonies using molecular identification.

Longer term maintenance of the newly isolated bacterium from DUWL 9

Pure cultures of *S. marcescens* from DUWL 9 were maintained on R2A and Nutrient agar. This bacterium produced white coloured colonies on both R2A and Nutrient agar medium at all temperatures tested when sub-cultured from GVPC medium. No pigmented colonies were observed. A unique identification code (UL 234 14) has been assigned to *S. marcescens* and freeze-dried stocks are stored at 4°C at our academic institutre.

The effect of Alpron™ on *S. marcescens* from clinical DUWL output water

Pure cultures of *S. marcescens* and the accompanying *P. aeruginosa* at $1x10^8$ cfu/ml showed that these bacteria were killed by Alpron™ at the manufacturers recommended treatment levels as there was no recovery after 7 days of incubation under laboratory conditions (p = 0.0001).

Growth statistics for *V. vermiformis*

As anticipated, *V. vermiformis* grew well on all freshly prepared, live bacterial feeds: *E. coli* (p = 0.0001), *S. marcescens* (p = 0.0001), *P. aeruginosa* (p = 0.0001) using one-way ANOVA.

Transmission electron microscopy (TEM)

V. vermiformis grown on pure freshly grown live bacteria

To determine whether *V. vermiformis* supported growth of the Gram negative *S. marcescens* (from DUWL 9, Fig. 2a) and two laboratory strains *E. coli* (XL blue, Fig. 2b) and *P. aeruginosa* (Fig. 2c) samples of *V. vermiformis* cells were examined for internalised bacterial cells within the cytoplasm and/or within their encysted form, using high resolution electron microscopy. Neither the trophozoidal amoebae nor their encysted forms produced metabolically active bacterial cells within their cell bodies with the exception of the occasional *V. vermiformis* cell that fed on *P. aeruginosa* (Fig. 2c, box).

Discussion

Water supports all forms of life within complex biomes in which, the established biofilm microbes vary considerably, in number and range. The physiological condition of the planktonic consortium is also constantly changing within the biofilm. Many bacteria possess a variety of virulence factors which, upon entry to a human host, can challenge the health of that individual. Those most at risk of infection are patients and practitioners

with immunocompromised status such as the elderly, HIV patients, smokers, alcohol/drug addicts, diabetics and sufferers of chronic lung diseases, heart disease and renal disease. This study set out to characterise a risk factor associated with pathogenic bacteria commonly found in DUWL systems alongside free living amoebae that graze on these bacteria. In some instances, ingested pathogenic bacteria manipulate the amoebal host for their own survival and multiplication, potentially leading to the death of their infected human host [43]. Lawsuits can be brought against the dental practitioner if causal links between an infection and the dental treatment water are confirmed [8]. Thus the importance of improving the quality of dental treatment water is essential, clinically, ethically and financially, to halt spread of disease from DUWL water to humans. Although infection rates in humans are generally minimal [23], continued preventative measures must be taken to decrease the possibility of contracting disease from contaminated DUWL output water.

V. vermiformis dominates the DUWL environment [7] and their initial introduction into this interventional device is likely to come from fresh water supplies used for the reservoir. The same source of water, which may supply domestic and clinical service providing premises, will also have nosocomial pathogens and amoebae prevalent within them [18, 21, 25, 34, 53], However, *Hartmanella* (*Vermiformis*) species are also reported to have been isolated from the throat of humans from as long ago as 1967 [54] implying that the high prevalence of *V. vermiformis* in the DUWL could also come from humans. This study, identified a clinical isolate of *S. marcescens*, from one out of 31 DUWL water samples. As *V. vermiformis* is a much more cosmopolitan feeder than many other free living amoebae [55] this means that there is a greater likelihood of a pathogenic bacterium utilising this taxon of amoeba as an effective means of transport and dispersal in this environment [40]. Previous work on grazing habits of *V. vermiformis* [14] indicated that small sized bacteria, from the simulated DUWL biofilm, were favoured as a food source.

Since the molecular identity of the bacterium from DUWL 9 was identified as *S. marcescens*, which is a typical nosocomial bacterium, considered to be a clinical isolate its likely survival in the clinical DUWL may have been related to the development of biocide resistance [27, 28]. Since the dental biocide used to treat the DUWL from which the water sample was taken was Alpron™, and its efficacy was tested in the laboratory on the isolated strain of *S. marcescens*. The results demonstrated that this dental biocide was effective on pure cultures of *S. marcescens* in the laboratory up to 1×10^8 cfu/ml. In a previous feasibility study [38], it was found that despite being treated with the same biocide, the planktonic bacterial counts of aerobic mesophilic bacteria were significantly higher in some DUWLs than those set for dentistry by government authorities. Other studies have demonstrated that a consortium of biofilm organisms is capable of surviving antibacterial agents at higher

inoculum levels [4, 15, 33]. However, *S. marcescens* is prone to develop multidrug resistance, via spontaneous mutations in the efflux pump genes, under high concentrations of biocide usage [27, 28]. Our results failed to confirm biocide resistance in *S. marcescens* under laboratory conditions suggesting the extracellular polymeric matrix environment of the biofilm provided protection from Alpron™ within the DUWL.

An earlier investigation suggested a permissive role of *H. vermiformis* (now called *Vermamoeba vermiformis*) for *P. aeruginosa* [12]. *P. aeruginosa* was the organism responsible for the introduction of control measures in dentistry after reports that it caused serious health problems to patients following dental treatment [26]. This study also explored the likelihood of *V. vermiformis* supporting the life cycle of *P. aeruginosa* under laboratory conditions.

The results of this study demonstrate that *V. vermiformis*, which was fed on *E. coli* and *P. aeruginosa* (p = 0.0001), grew to the same extent as it did on the non-pigmented *S. marcescens* isolated from clinical DUWL water (p = 0.0001). These results agree with those of Singh [49] in which free living amoebae were fed only on a non-pigmented *S. marcescens*. However, in this investigation, *V. vermiformis* also fed on *P. aeruginosa* (p < 0.05). These results strongly agree with the study conducted by Pickup et al. [39], but disagrees with those of Groscop & Brent [19] who suggested that *P. aeruginosa* was toxic to an unknown species of the genus *Hartmannella*.

S. marcescens is a known nosocomial pathogen and can cause a variety of infections in humans including blindness in the susceptible host [17, 20, 52]. It is thus important to understand its proliferative mechanisms in relation to its existence in the DUWL environment to inform the future development of disinfection regimes. Since no evidence for bacterial cells within the encysted amoebae was observed by ultrastructure, this suggests that *V. vermiformis* is not acting as a vector to support the proliferation of the nosocomial pathogen *S. marcescens* and disagrees with Cateau et al. [12] for *P. aeruginosa*, although strain differences may apply.

During the past few decades, infection control procedures in dentistry have changed significantly. The basis of dental infection control is to create and maintain a safe clinical environment and to remove, or reduce, the risk of disease transmission as much as possible to patients and dental health care workers. This study confirms that, despite the recommended and appropriate control measures being employed, bacteria such as *S. marcescens* can still be isolated in the laboratory from clinical DUWL water. Care must be taken to use biocides according to manufacturer's instructions to avoid multidrug resistance taking place. In addition, it is also important to adhere to the regular purging protocols recommended by the manufacturers' of the biocide.

Our investigation confirms that *V. vermiformis* can actively feed on fresh *P. aeruginosa* and *S. marcescens*, both are small-sized bacteria of which the latter was isolated in this study. This is in agreement with the description of an ideal food source for amoebae suggested by Pickup et al. [39] i.e ease of intake during phagocytosis/ingestion. Since amoebae appear to be genetically programmed to eat bacteria it is plausible to suggest that *V. vermiformis*, may be able to control bacterial populations by feeding on newly dividing *S. marcescens* providing a promising outcome for infection control in dental treatment.

Recommendations

- To avoid operator failure, make sure the responsible staff knows the treatment regime of the biocide used for their DUWLs (when to purge and working concentrations for purging and for daily treatment use).

- Keep log of date of purging and how long the DUWL has been exposed to disinfectant.

- Use biocides within their use-by-date.

References

1. Al Shorman H, Nabaa LA, Coulter WA, Pankhurst CL, Lynch E (2002) Management of dental unit water lines. Dent Update 29: 292-298.

2. American Dental Association (ADA) (2004) Statement on Dental Unit Waterline. http://www.ada.org/1856.aspx. Accessed 25 April 2015.

3. Anon (1993) Council Directive 93/42/EEC of 14 June 1993 concerning medical devices. OJEUL 169: 1-43.

4. Anwar H, Costerton JW (1990) Enhanced activity of combination of tobramycin and piperacillin for eradication of sessile biofilm cells of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 34: 1666-1671.

5. Atlas RM, Williams JF, Huntington MK (1995) *Legionella* contamination of dental-unit waters. Appl Environ Microbiol 61: 1208-1213.

6. Barbaree JM, Fields BS, Feeley JC, Gorman GW, Martin WT (1986) Isolation of protozoa from water associated with a Legionellosis outbreak and demonstration of intracellular multiplication of *Legionella pneumophilia*. Appl Environ Microbiol 51: 422-424.

7. Barbeau J, Buhler T (2001) Biofilms augment the number of free-living amoebae in dental unit water lines. Res Microbiol 152: 75-760.

8. Barbeau J. (2007) Lawsuit against a dentist related to serious ocular infection possibly linked to water from a dental handpiece. J Can Dent Assoc 73: 618-622.

9. Barbot V, Robert A, Rodier M, Imbert C (2012) Update on infectious risks associated with dental unit waterlines. FEMS Immunol. Med Microbiol 65: 196-204.

10. Barbot V, Costa D, Deborde M, Imbert C (2014) Efficacy of dental unit disinfectants against *Candida* spp. and *Hartmannella vermiformis*. Pathog Dis 70(3): 289-296.

11. Blake GC (1963) The incidence and control of bacterial infection in dental spray reservoirs. Br Dent J 115: 413-416.

12. Cateau E, Imbert C, Rodier MH (2008) *Hartmannella vermiformis* can be permissive for *P. aeruginosa*. Lett Appl Microbiol 47: 475-477.

12. Chate RA (2010) An audit improves the quality of water within the dental unit water lines of general dental practices across the East of England. Br Dent J 209(7): E11.

13. Dillon A, Achilles-Day U, Singhrao SK, Pearce M, Morton LHG, Crean S (2014[a]) Biocide sensitivity of *Vermamoeba vermiformis* isolated from dental-unit-waterline systems. Int Biodeter Biodegr 88: 97-105.

14. Dillon A, Singhrao SK, Achilles-Day U, Pearce M, Morton LHG, Crean S (2014[b]) *Vermamoeba vermiformis* does not propagate *Legionella pneumophila* subsp. Pascullei in a simulated laboratory dental-unit-waterline system. Int Biodeter Biodegr 90: 1-7.

15. Domingue G, Ellis B, Dasgupta M, Costerton JW (1994) Testing antimicrobial susceptibilities of adherent bacteria by a method that incorporates guidelines of the National Committee for Clinical Laboratory Standards. J Clin Microbiol 32: 2564-2568.

16. D'Ovidio C, Carnevale A, Pantaleone G, Piattelli A, Di Bonaventura G (2011) First report of an acute purulent maxillary sinusitis caused by *Pseudomonas aeruginosa* secondary to dental implant placement in an immunocompetent patient. Br Dent J 211: 205-207.

17. Equi RA, Green WR (2001) Endogenous *Serratia marcescens* endophthalmitis with dark hypopyon: case report and review. Surv Ophthalmol 46 (3): 259-268.

18. Fields BS, Fields SR, Chin Loy JN, White EW, Steffens WL, Shotts EB (1993) Attachment and entry of *Legionella pneumophila* in *Hartmannella vermiformis*. J Infect Dis 167: 1146-1150.

19. Groscop JA, Brent MM (1964) The effects of selected strains of pigmented microorganisms on small free-living amoebae. Can J Microbiol 10: 579-584.

20. Hejazi A, Falkiner FR (1997) *Serratia marcescens*. J Med Microbiol 46(11): 903–912.

21. Henke M, Seidel KM (1986) Association between *Legionella pneumophila* and amoebae in water. Isr J Med Sci 22: 690-695.

22. Kohn W, Collins A, Cleveland J, Harte J, Eklund K, Malvitz D (2003) Guidelines for infection control in dental health-care settings. MMWR. Recommendations And Reports / Centers for Disease Control [serial online] 52(RR-17): 1-61. http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5217a1.htm. Accessed 1 May 2015.

23. Lal S, Singhrao SK, Bricknell M, Pearce M, Morton LHG, Ahmed W, Crean S (2014) Monitoring dental unit water line output water by current in-office test kits. Curr Microbiol 69(2):135-142.

24. Langeland K (1972) Prevention of pulpal damage. Dent Clin North Am 16: 709-732.

25. Marciano-Cabral F, Jamerson M, Kaneshiro ES (2010) Free-living amoebae, *Legionella* and *Mycobacterium* in tap water supplied by a municipal drinking water utility in the USA. J Water Health 8(1): 71-82.

26. Martin M (1987) The significance of the bacterial contamination of dental unit water systems. Br Dent J 163: 152-154.

27. Maseda H, Hashida Y, Konaka R, Shirai A, Kourai H (2009) Mutational upregulation of a resistance-nodulation-cell division-type multidrug efflux pump, SdeAB, upon exposure to a biocide, cetylpyridinium chloride, and antibiotic resistance in *Serratia marcescens*. Antimicrob Agents Chemother 53(12): 5230-5235.

28. Maseda H, Hashida Y, Shirai A, Omasa T, Nakae T (2011) Mutation in the sdeS gene promotes expression of the sdeAB efflux pump genes and multidrug resistance in *Serratia marcescens*. Antimicrob Agents Chemother 55(6): 2922-2926.

29. McEntegart MG, Clark A (1973) Colonisation of dental units by water bacteria. Br Dent J 34: 140-142.

30. Michel R, Just HM (1984) Acanthamoebae, Naegleria and other free-living Amoebae in cooling and rinsing water of dental treatment units. Zentralbl Bakteriol Mikrobiol Hyg B 179: 56-72.

31. Michel R, Borneff M (1989) The significance of amoebae and other protozoa in water conduit systems in dental units. Zentralbl Bakteriol Mikrobiol Hyg B 187: 312-323.

32. Miles AA, Misra SS (1938) The estimation of the bactericidal power of the blood. J Hyg 38: 732-749.

33. Moskowitz SM, Foster JM, Emerson J, Burns JL (2004) Clinically feasible biofilm susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis. J Clin Microbiol 42: 1915-1922.

34. Nazar M, Haghighi A, Taghipour N, Ortega-Rivas A, Tahvildar-Biderouni F, Nazemalhosseini Mojarad E, Eftekhar M (2012) Molecular identification of *Hartmannella vermiformis* and *Vannella persistens* from man-made recreational water environments, Tehran, Iran. Parasitology Res 111: 835-839.

35. Oppenheim BA, Sefton AM, Gill ON, Tyler JE, O'Mahony MC, Richards JM, Dennis PJ, Harrison TG (1987) Widespread *Legionella pneumophila* contamination of dental stations in a dental school without apparent human infection. Epidemiol Infect 99: 159-166.

36. Pankhurst CL, Philpott-Howard JN (1993) The microbiological quality of water in dental chair units. J Hosp Infect 23: 167-174.

37. Pankhurst CL, Johnson NW, Woods RG (1998) Microbial contamination of dental unit waterlines: the scientific argument. Int Dent J 48: 359-368.

38. Pearce M, O'Donnell H, Singhrao SK, Crean S (2013) Assessing water discharged from dental units using Dip Slides[TM]. The British Society for Oral and Dental Research (BSODR) abstract number 181571.

39. Pickup Z, Pickup R, Parry, JD (2007[a]) Effects of bacterial prey pecies and their concentration on growth of the Amoebae *Acanthamoeba castellanii* and *Hartmannella vermiformis*. Appl Environ Microbiol 73: 2631-2634.

40. Pickup Z, Pickup R, Parry JD (2007[b]) A comparison of the growth and starvation responses of *Acanthamoeba castellanii* and *Hartmannella vermiformis* in the presence of suspended and attached Escherichia coli K12. FEMS Microbiol Ecol 59: 556-563.

41. Porteous NB, Redding SW, Jorgensen JH (2004) Isolation of non-tuberculosis *Mycobacteria* in treated dental unit waterlines. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 98: 40-44.

42. Rice EW, Rich WK, Johnson CH, Lye DJ (2006) The role of flushing dental water lines for the removal of microbial contaminants. Public Health Rep. 121: 270-274.

43. Ricci ML, Fontana S, Pinci F, Fiumana E, Pedna MF, Farolfi P, Sabattini MA, Scaturro M (2012) Pneumonia associated with a dental unit waterline. Lancet 379: 684.

44. Rowland BR, Voorheesville NY (2003) Bacterial contamination of dental unit waterlines: What is your dentist spraying into your mouth. Clin Microbiol Newsl 25(10): 73-77.

45. Salah IB, Ghigo E, Drancourt M (2009) Free-living amoebae, a training field for macrophage resistance of mycobacteria. Clin Microbiol Infect 15(10): 894-905.

46. Sandstrom G, Saeed A, Abd H (2010) *Acanthamoeba polyphaga* is a possible host for *Vibrio cholerae* in aquatic environments. Exp Parasitol 126(1): 65-68.

47. Schulze-Robbecke R, Feldman C, Fischeder R (1995) Dental units: an environmental study of sources of potentially pathogenic *Mycobacteria*. Tuber Lung Dis 76: 318-323.

48. Siegel SC, von Fraunhofer JA (2002) The effect of hand piece spray patterns on cutting efficiency. J Am Dent Assoc 133: 184-188.

49. Singh B N (1942) Toxic effects of certain bacterial metabolic products on soil protozoa. Nature 149: 168.

50. Singh T, Coogan MM (2005) Isolation of pathogenic *Legionella* species and Legionella-laden amoebae in dental unit waterlines. J Hosp Infect 61: 257-262.

51. Stanley HR (1971) Pulpal response to dental techniques and materials. Dent Clin North Am 15: 115-126.

52. Tan N, Galvante, PR, Chee SP (2014) Endogenous *Serratia marcescens* endophthalmitis: an atypical presentation. Eye (Lond) 28(1): 108-109.

53. Wadowsky RM, Butler LJ, Cook MK, Verma SM, Paul MA, Fields BS, Keleti G, Sykora JL, Yee RB (1988) Growth supporting activity for *Legionella pneumophila* in tap water cultures and implication of Hartmannellid amoebae as growth factors. Appl Environ Microbiol 54: 2677-2682.

54. Wang SS, Feldman HA (1967) isolation of *Hartmannella* species from human throats. N Engl J Med 277: 1174-1179.

55. Weekers PHH, Bodelier PLE, Wijen JPH, Vogels GD (1993) Effects of grazing by the free-living soil amoebae *Acanthamoeba castellanii*, *Acanthamoeba polyphaga*, and *Hartmannella vermiformis* on various bacteria. Appl and Environ Microbiol 2317-2319.

56. Wenzel RP (1994) Healthcare workers and the incidence of nosocomial infection: can treatment of one influence the other?—a brief review. J Chemother 4: 33-37.

57. Williams JF, Johnston AM, Johnson B, Huntington MK, Mackenzie CD (1993) Microbial contamination of dental unit waterlines: prevalence, intensity and microbiological characteristics. J Am Dent Assoc 124: 59-65.

58. Williams HN, Paszko-Kolva C, Shahamat M, Palmer C, Pettis C, Kelley J (1996) Molecular techniques reveal high prevalence of Legionella in dental units. J Am Dent Assoc 127: 1188-1193.

59. Winiecka-Krusnell J, Wreiber K, von Euler A, Engstrand L, Linder E (2002) Free-living amoebae promote growth and survival of *Helicobacter pylori*. Scand J Infect Dis 34(4): 253-256.

Legends

Fig. 1 Phase contrast image of *Vermamoeba vermiformis*. Small newly emerging trophozoite and smooth spherical cysts.

Fig. 2 Transmission electron micrographs of encysted *V. vermiformis* after feeding on *S. marcescens* (Fig. 2a), *E. coli* (XL blue) (Fig. 2b) and *P. aeruginosa* (Fig. 2c). No bacterial cells were observed within the amoebae or their encysted forms except, for one amoebal cell fed on *P. aeruginosa* (Fig. 2c, box).