Colonization by *Pseudomonas aeruginosa* of dental unit waterlines and its relationship with other bacteria: suggestions for microbiological monitoring

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ABSTRACT

Pseudomonas aeruginosa is an environmental bacterium, ubiquitous in aquatic habitats and water distribution systems, including dental unit waterlines (DUWLs). We investigated the prevalence of *P. aeruginosa* in DUWLs from private dental settings. We also analyzed the relationship between *P. aeruginosa* contamination and the presence of *Legionella* spp. and total viable count (TVC) in order to suggest a simple and inexpensive protocol to test the quality of water from DUWLs. We detected and quantified *P. aeruginosa* both by culture and by a PMA (propidium monoazide)-qPCR method. Overall, we detected *P. aeruginosa* in 17 samples using the PMA-qPCR and in 11 samples using the culture. All culture-positive samples were positive with the PMA-qPCR too, with an agreement between the two methods of 93% and a Cohen's kappa coefficient of $\kappa = 0.747$ (good concordance). Comparing results with results of our previous study, we noted that (a) *P. aeruginosa* was isolated only from DUWLs with high TVC and (b) five out of six *Legionella*-positive samples were negative for *Pseudomonas* spp. Our final suggestion is that the cleanliness of DUWLs should be assessed by TVC because it is a good indicator of the presence of pathogens such as *Legionella* spp. and *P. aeruginosa*.

Key words | dental setting, enumeration, *Pseudomonas* spp., quantitative real-time PCR, total viable count

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INTRODUCTION

The dental unit waterlines (DUWLs) in a dental chair unit (DCU) consist of approximately 6 m of narrow-bore (2 mm internal diameter) flexible polyurethane or polyvinyl chloride plastic tubing connected by brass or non-flexible plastic couplings (Walker & Marsh 2007). The high surface area-to-water volume ratio and the intermittent use of DUWLs, leading to stagnation of water for extended periods, promote the formation of microbial biofilm within DUWLs (Barbot *et al.* 2012).

Microorganisms may access DUWLs through incoming municipal water, contaminated independent water reservoirs (e.g. water bottles) or retrograde movement of output water and saliva into dental handpieces (Tilak *et al.* 2012). Once the biofilm has been established, individual microorganisms and pieces of biofilm can detach and seed into dental output water (Szymanska 2003).

The bacterial species recovered from the samples taken from dental chairs were all Gram-negative and belonged to families of aquatic and soil bacteria that exhibit very low pathogenicity; however, the risk of contaminated DUWLs is most significant for *Legionella* spp., non-tuberculous mycobacteria (e.g. *Mycobacterium abscessus*) and *Pseudomonas aeruginosa*,

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especially for immunocompromised patients and medically compromised individuals (Martin 1987), and for dental staff (Reinthaler & Mascher 1986).

The first evidence of microbial contamination in dental output water was recognized by Blake (1963); following this seminal publication, further studies have identified a wide variety of bacterial, fungal and protozoan microorganisms colonizing DUWLs, including nosocomial pathogens: *L. pneumophila*, non-tuberculous *Mycobacterium* spp. and *P. aeruginosa* (Szymańska *et al.* 2008). The primary route of transmission for most of these pathogens from DUWLs is aerosolization of the output water via dental handpieces and subsequent inhalation of airborne droplets (Pankhurst 2003). However, more rarely, microorganisms may be transmitted by imbibing or contamination of wounds (Pankhurst & Coulter 2007).

The formation of mixed communities in an environmental niche, such as DUWLs, is determined by a mutual influence of exoproducts: Qin *et al.* (2009) indicated that supernatant from *P. aeruginosa* inhibited *Staphylococcus epidermidis* growth in planktonic cultures. They also found that *P. aeruginosa* extracellular products, mainly polysaccharides, disrupted established *S. epidermidis* biofilms.

P. aeruginosa produces virulence factors and exometabolites, such as proteases, haemolysins, blue phenazine pyocyanin (PCN) and the fluorescent green siderophore pyoverdin (Pvd) (Smith & Iglewski 2003; Irie *et al.* 2005). PCN is a blue, secondary metabolite with the ability to oxidize and reduce other molecules and therefore can kill microbes competing against *P. aeruginosa* and enhanced *P. aeruginosa* survival in a highly populated environment (Hassan & Fridovich 1980; Jimenez *et al.* 2012). Pvd confers benefits to *P. aeruginosa* for adaptation to environments. Pvd are important virulence factors and are required for pathogenesis: providing a crucial nutrient (i.e., iron), regulating virulence factors (including exotoxin A and the protease PrpL), supporting the formation of biofilms, limiting the growth of other bacterial species (and serving as a sort of antimicrobial) by limiting iron availability, and sequestering other metals and preventing their toxicity (Lamont *et al.* 2002; Banin *et al.* 2005).

Pseudomonas strains were found to secrete anti-*Legionella* compounds. This anti-*Legionella* activity was correlated with the secretion of biosurfactants (Berjeaud *et al.* n.d.), classified in two families: lipopeptides and rhamnolipids. Interestingly, these compounds were found to display antimicrobial activity towards all of the *Legionella* spp. tested. Moreover, the activities of these molecules were shown to be higher towards *Legionella* spp. than all of the other Gram-positive or Gram-negative bacteria assayed. *In vitro* studies of bacterial–fungal coinfection have shown that the growth of *Candida albicans* and biofilm development of *Cryptococcus neoformans*, *Aspergillus fumigatus* and *Trichophyton* spp. are inhibited by *P. aeruginosa* phenazine toxins or quorum-sensing molecules (Gibson *et al.* 2009; Bandara *et al.* 2010; Mowat *et al.* 2010; Rella *et al.* 2012).

The aim of this study is to investigate the prevalence of *P. aeruginosa* in DUWLs and tap water samples from private dental settings using PMA (propidium monoazide)-qPCR and standard culture methods in order to compare their suitability as methods for the detection and enumeration of *P. aeruginosa* in dental settings.

In addition, this study also investigates the relationship between *P. aeruginosa* and *Legionella* contamination and the total viable count (TVC) of aerobic heterotrophic bacteria based on the data acquired so far and on critical issues observed in a previously published study (Ditommaso *et al.* 2016) in order to suggest a simple and inexpensive protocol to test the water from the DUWLs.

MATERIALS AND METHODS

Water samples taken from DUWLs and sink faucets (tap water) of 26 private dentistry settings in 2015, as previously described (Ditommaso *et al.* 2016), underwent microbiological examination.

Pseudomonas aeruginosa quantification by the culture method

We detected and quantified *P. aeruginosa* by the culture according to the ISO 16266 method (International Standard Organization 2006). Briefly, we filtered 250 ml of the water samples through a sterile cellulose ester membrane filter with a rated pore diameter equivalent to 0.45 μm (Millipore). The filter membrane was aseptically placed on a Petri dish containing Cetrimide agar (Thermo Scientific™) and

incubated at 36 ± 2 °C for 44 ± 4 h. All of the colonies that produced a blue/green (pyocyanin) colour were confirmed *P. aeruginosa*: we submitted all other colonies (fluorescent, but not blue/green, and reddish brown pigmented) for confirmatory tests according to ISO 16266.

Viable *Pseudomonas aeruginosa* quantification by PMA-qPCR

We analyzed extracted genomic DNA by the Primerdesign™ Genesig® commercial kit for *P. aeruginosa* (Genesig, PrimerDesign Ltd). This kit contains reagents to amplify and quantify (using TaqMan chemistry) an approximately 100 bp fragment from the target gene regA, which encodes a *P. aeruginosa* toxin, a synthesis regulating gene. The kit is designed to have the broadest detection profile possible, while remaining specific to the *P. aeruginosa* genome. The primers and probe sequences in this kit have 100% homology with a broad range of *P. aeruginosa* sequences based on a comprehensive bioinformatics analysis. We constructed a standard curve using a stock solution of *P. aeruginosa* genomic DNA (contained in the kit) titrated at 2×10^5 DNA copies μl^{-1}. The kit also supplies exogenous plasmid DNA, which is used as an internal control, and a separate primer–probe mix to monitor any inhibitory effects that may take place in the reaction mix. Under optimal polymerase chain reaction (PCR) conditions, the method can detect less than 100 copies of the target template.

To discriminate between the DNA of viable and nonviable *P. aeruginosa* cells, we implemented a pretreatment of the samples with PMA in conjunction with the qPCR technique (Ditommaso *et al.* 2015).

Statistical analysis

We analyzed the qPCR data using the Opticon Monitor Analysis Software version 3.4 (Bio-Rad, Hercules, CA, USA). The Mantel–Haenszel chi-square test of trend was applied to evaluate the association between TVC and *P. aeruginosa*. The proportions were compared using Fisher's exact test. An independent-measures analysis of variance (ANOVA) test was used to compare the *Legionella* spp. mean counts obtained by the molecular method from samples with and without *Pseudomonas* spp.

RESULTS

We took 26 samples from tap water and 60 samples from DUWL, and we compared the results obtained using the culture and PMA-qPCR methods.

Overall, using both methods, we found *Pseudomonas* spp. in 23 samples. In one DUWL sample, we detected either *P. aeruginosa* or *P. putida* using the culture method; in another DUWL sample, we detected both *P. aeruginosa* and *P. fluorescens*.

Out of these 23 samples, we found *P. aeruginosa* in 17 samples using the PMA-qPCR method, whereas in only 11 samples using the culture method. All *P. aeruginosa*-positive samples from the culture method were positive in the PMA-qPCR method and, of these, 100% were collected from the output water of DUWLs (Table 1).

The agreement between the two methods was 93%: we also calculated the Cohen's kappa coefficient that shows a good concordance ($\kappa = 0.747$).

The *P. aeruginosa* counts in 14 DUWL samples determined using PMA-qPCR ranged from 32 to 2.1×10^4 Genetic Units/L (GU/L) (geometric mean: 1.1×10^3 GU/L). The GU/L values for the three tap water samples ranged from 32 to 2.5×10^2 (geometric mean: 81 GU/L).

We isolated *P. aeruginosa* only from dental unit samples with bacterial loads ranging from 10^2 CFU/mL to $>3 \times 10^3$ CFU/mL (Table 2).

One of the 20 tap water samples that satisfied the required European Council Directive 98/83/EC Standards for drinking water and two samples with bacterial loads ranging from 10^2 to 10^3 CFU/mL were contaminated with *P. aeruginosa*; however, the *P. aeruginosa* counts in three tap water samples ranged from 32 to 2.5×10^2 GU/L.

Table 1 | Agreement between PMA-qPCR and culture methods

	Culture		PMA-qPCR	
Type of sample	**Positive (*n*)**	**Negative (*n*)**	**Positive (*n*)**	**Negative (*n*)**
Tap water	0	26	3	23
DUWL output	11	49	14	46
Total *n* (%)	11 (12.8%)	75 (87.2%)	17 (19.8%)	69 (80.2%)

Observed concordance = 93%; Cohen's $\kappa = 0.747$ (good).
Culture method: sensibility = 65%; specificity = 100%; PPV = 100%; NPV = 92%.

Table 2 | Relationship between *P. aeruginosa*-positive PMA-qPCR and the TVCs

TVCs	DUWL water samples (*n*)	*P. aeruginosa* PMA-qPCR (*n*)	Tap water samples (*n*)	*P. aeruginosa* PMA-qPCR (*n*)
Drinking water threshold[a]	3	0	20	1
10^2–3×10^3 CFU/mL	31	3	5	2
$>3 \times 10^3$ CFU/mL	26	11	1	0
Total	60	14	26	3

[a]Threshold values established by the European Council Directive 98/83/E: <20 CFU/mL at 36 °C and <100 CFU/mL at 22 °C (Mantel–Haenszel chi-square test for trend = 7,18, $p = 0.007386$).

A chi-square test proved a statistically significant association between heterotrophic bacterial contamination and the presence of *P. aeruginosa* colonization in the DUWLs.

Interestingly, by comparing these results with the results obtained in a previous study (*Legionella* spp. were detected in all samples by the PMA-qPCR test, and only 7% (6/86) were positive using the culture method) (Ditommaso *et al.* 2016), we noted that five out of six samples that were positive for *Legionella* by culture and were negative for *Pseudomonas* spp. in both the culture and the molecular methods. However, the differences between the number of *Legionella*-positive samples recovered from the two groups (*Pseudomonas* spp.-positive and -negative samples) were not significant at $p < 0.05$ (Fisher's exact test) (Table 3).

The *Legionella* spp. counts determined by the molecular method from samples without *Pseudomonas* spp. were greater than those from samples with *Pseudomonas* spp. (Table 4); however, the differences were not significant at the 95% confidence level (two-way ANOVA: $p = 0.29$).

Table 3 | Comparison of results for the presence or absence of *Legionella* as determined by the culture assay and the presence or absence of *Pseudomonas* spp. determined by the PMA-qPCR assay and the culture method

		***Pseudomonas* spp. (both methods)**	
		Positive (*n*)	**Negative (*n*)**
Legionella spp. culture	Positive (*n*)	1	5
	Negative (*n*)	22	58
	Total (*n*)	23	63

Fisher's exact test = 1; $p > 0.05$.

Table 4 | Comparison of results for the presence or absence of *Pseudomonas* spp. as determined by culture and by the PMA-qPCR assay and the amount of *Legionella* spp.

	***Legionella*-positive samples (*n*)**	**Geometric mean (GU/L ± SD)**
Pseudomonas spp.-positive samples	23	$3.8 \times 10^4 \pm 4.3 \times 10^5$
Pseudomonas spp.-negative samples	63	$6.2 \times 10^4 \pm 1.2 \times 10^6$

DISCUSSION

The bacterial species recovered from the samples taken from the dental chairs were all Gram-negative and belonged to the families of aquatic and soil bacteria (Barbeau *et al.* 1998; Ditommaso *et al.* 2018) and some of these, including *Pseudomonas* spp., are opportunistic human pathogens (Hsueh *et al.* 1998; Brooke 2012; Orsini *et al.* 2014).

In acute infections, *P. aeruginosa* traverses the mucosa, provoking bacteremia and infecting distant organs. For these reasons, bacteria mostly transmigrate at the level of the lung alveoli (Williams *et al.* 2010) and the urinary tract (Mittal *et al.* 2009), composed of a single layer or transitional epithelia, as well as the cornea, composed of a small number of non-keratinized cell layers (Fleiszig & Evans 2010).

The clinical importance of the presence of non-mucoid strains of *P. aeruginosa* in DUWLs is not known, and the risk estimates by a quantitative microbial risk assessment (QMRA) are prohibitively complex and not applicable. The World Health Organization stated that 'it is not possible to consider all water-related human pathogens in a QMRA' (World Health Organization 2016). This statement applies

especially to microorganisms that have different transmission routes and different target organs. In the case of *Pseudomonas* spp., the infection may be acquired by ingestion, inhalation of contaminated aerosols and aspiration of contaminated water of DUWLs and, according to the exposure route, can cause different clinical presentations: oral abscesses (Martin 1987), pneumonia (Stryjewski & Sexton 2003).

Moreover, the high variability of water/aerosols exposure frequency and high variability of *P. aeruginosa* populations in terms of infectivity, and the different susceptibility of the host, makes it difficult to establish the dose infection threshold. As reported by Bentham & Whiley (2018), these represent the critical points that make the QMRA difficult also for *Legionella* spp.

Immunodeficient patients are at a greater risk of acquiring opportunistic 'non-oral-pathogen-related' periodontitis (Slots *et al.* 1990). Previously, Martin (1987) reported the cases of two immunodeficient patients who developed localized *P. aeruginosa* infections following dental restorations, suggesting that the dental unit water was the source of these infections: Martin demonstrated that the two immunocompromised patients had the same pyocin types as isolates recovered from dental unit water.

In previously published studies, tests of water from DUWLs for *P. aeruginosa* showed that the prevalence of this bacterial species in DCUs varied from 3.8% (Walker *et al.* 2004) to 24% (Schulze-Röbbecke *et al.* 1995).

In this study, the culture-based method, which is the most commonly prescribed approach for monitoring *P. aeruginosa* in drinking water, detected the bacteria in 12.8% of all of the samples taken from air–water syringes and turbines (combined), while the PMA-qPCR tests showed that this bacterial species was present in 19.8%. The use of PMA avoided an overestimation of bacterial prevalence, as it limited the detection to only vital *P. aeruginosa* cells. Either *P. putida* or *P. fluorescens* were detected using the culture-based method.

Since good agreement between the culture method and the molecular method was observed (Cohen's index $\kappa = 0.747$), it can be concluded that the classic culture method, which is less expensive and more feasible in all microbiology laboratories, can be used to detect this pathogen in the environmental matrix. This finding is in contrast to the observation in a previous study (Ditommaso *et al.* 2016) in which the target microorganism was *Legionella*: *Legionella* spp. were detected in 100% of the samples using the PMA-qPCR method, whereas these bacteria were detected in only 7% of the samples using the culture method. This discrepancy can be explained by the different nutritional/environmental and biological needs of the two pathogens.

Legionella cells can be found in many types of water systems such as plumbing systems, hot-water tanks, cooling towers, evaporative condensers of large air-conditioning systems, and hot tubs. In these environments, *Legionella* is generally found associated with amoebae and/or biofilms. The interactions that may occur between the microbial flora and *Legionella* in these aquatic communities are important in regard to the regulation of population dynamics in bacterial ecosystems, particularly by the production of antimicrobial compounds. *Legionella* uses amoebae to escape stressful living conditions, e.g., the presence of biocides, low water temperature, or the presence of pyocyanins produced by *Pseudomonas*, and acquires the feature of being viable but not cultivable (VBNC). Other bacteria, such as *Legionella*, may be either a temporary non-culturable state or may represent the majority of the microflora from many natural habitats that remain 'as yet uncultured' (Barer & Harwood 1999).

It is known that some strains of *P. aeruginosa* produce bacteriocins (Govan 1986) that can inhibit the growth of other microorganisms. This ability could confer a competitive advantage to *P. aeruginosa* in colonization of the DUWLs. Moreover, *P. aeruginosa* can mask the presence of *Legionella* spp., as reported by Rowland (2003); therefore, the non-cultivatable bacteria should be detected with the molecular method. Furthermore, the method for enumerating *Legionella* in water samples is less robust than that for *P. aeruginosa*, which provides for simple filtration and cultivation of bacteria on the same membrane placed on the selective medium.

Water supplied by 95% (57/60) of the DUWLs failed current European Union potable-water guidelines on microbial load (<20 CFU/mL at 36 °C and <100 CFU/mL at 22 °C), and 24.5% of these were *P. aeruginosa*-positive.

The statistically significant association between the presence of *P. aeruginosa* and high concentrations of

heterotrophic bacteria confirms that the total bacterial load is, in itself, a useful indicator of the need for remediation of dental units aimed at removing biofilms, which are a receptacle for many pathogenic microbial species, including species of *Legionella* and *Pseudomonas*.

Ineffective control measures (disinfection, anti-retraction devices, pre-treated water, in-line filtration, flushing and/or drying of dental units) probably lead to *P. aeruginosa* colonization, as demonstrated by the percentage of positive water samples (19.8%). A number of European surveys confirm that dentists have poor awareness of DUWL contamination and an inadequate understanding of how the microbial risk should be managed, although dentists are positively seeking more information and help in this regard (Kamma *et al.* n.d.; Robert *et al.* 2013; Szymańska & Sitkowska 2013).

Thus, even if the total microbial count does not always represent a risk for patients and healthcare workers, the presence of opportunistic pathogens such as *P. aeruginosa* and *Legionella* spp. may be dangerous.

However, *Legionella* spp. and *P. aeruginosa* infections are just two of a wide variety of bacterial, fungal and protozoal microorganism potentially fatal diseases associated with water-related infections. Exposure to pathogenic bacteria in buildings such as hospitals, hotels, dental settings, etc., is a known public health risk. Therefore, the legislative guidance documents and evidence-based guideline strategies, used to protect society from exposure to all pathogenic bacteria, must be constantly reviewed and revised. Furthermore, microbiological monitoring of DUWLs must be carried out where there is doubt about the efficacy of the control regime, or it is known that recommended temperature, disinfectant concentrations or other precautions are not being consistently achieved throughout the dental water system.

CONCLUSIONS

Our final suggestion is that the compliance with cleaning regimens of DCUs should be assessed by the use of routine microbiological testing for TVC in DUWL output water because contamination by these organisms is a good indicator of the potential presence of waterborne pathogenic bacteria. The most accurate method is to send dental treatment water samples to a laboratory; however, that is not always cost effective or practical. The most common way to monitor DUWLs is with commercially available in-office test kits. According to ADA publications, practitioners should periodically use in-office screening kits to monitor the quality of their dental unit water to ensure that waterline treatments are effective.

There are many testing devices that offer a quick, affordable (5–6 € test) and easy method of estimating the number of free-floating, heterotrophic bacteria in dental unit water, e.g., Millipore HPC Total Counter Sampler, 3M Petrifilm AC Plates and Pall Aquasafe Water Test Kit (Morris *et al.* 2010). These in-office screening kits do not need to yield results equal to those of the standard method (International Standards Organization 1999), which is considered the gold standard method, but they should yield similar results (Puttaiah *et al.* 2002; Bartoloni *et al.* 2006; Cohen *et al.* 2007; Momeni *et al.* 2012).

According to Morris *et al.* (2010), Petrifilm AC Plates (3M) provide a more accurate and easy to use alternative to the spread plate method using R2 agar culture (accuracy 87%; sensitivity 79%; specificity 98%; positive predictive values (PPV) 99%; negative predictive values (NPV) 76%).

If you use an in-office test kit, it would be advisable to send periodically (for example, once a year) water samples from the DUWLs to a microbiology laboratory for evaluation of aerobic heterotrophic bacteria count using standard methods.

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