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Dental unit water treatment with hydrogen peroxide and monovalent silver ions artificially contaminated with freshly isolated pathogens

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Key words: Dental unit water, hydrogen peroxide, silver ions, cross infection
Parole chiave: Riunito odontoiatrico, acqua, perossido di idrogeno, ioni argento, infezione crociata

Abstract

Background: *Dental unit water (DUW) could be contaminated by human pathogens coming from biological fluids penetrated during patient treatment and by opportunistic pathogens detached from aquatic biofilm. These microorganisms could be spread to following patients. We tested the disinfectant activity of hydrogen peroxide and monovalent silver ions (H_2O_2-Ag^+) into DUW artificially contaminated with freshly isolated pathogens.*
Methods: *The tested microorganisms were Staphylococcus aureus, Enterococcus faecalis, Candida albicans, Pseudomonas aeruginosa, Legionella pneumophila, Mycobacterium chelonae, non-pathogenic Bacillus clausii spores. Bacterial suspensions were inoculated into the waterlines of pre-sterilized dental turbines. The test-turbines were connected to DUW and contaminated water was treated for 10 minutes with H_2O_2-Ag^+-based disinfectant (H_2O_2 3% v/v, Ag^+ 0.001% w/v). The control-turbines were left untreated. Turbines were washed with sterile hard water used to assess the residual bacterial loads (expressed in colony forming units –cfu). Each strain was tested five times and the mean log loads were assessed. Following the European Standardization Committee, the disinfectant activity was evaluated as mean log load reduction, that is, the difference between the mean log load detected on the control-turbines and the mean log load detected on the test-turbines.*
Results*: Mean bacterial loads detected on the control-turbines ranged between 10^5-10^7 cfu. The mean log load reductions resulted 7.5 log cfu for S. aureus, E. faecalis, P. aeruginosa, 6.3 for C. albicans, 5.4 for L. pneumophila, 5.3 for M. chelonae, 2.9 for B. clausii spores.*
Conclusions: *DUW disinfection with H_2O_2-Ag^+ could help minimize the risk that planktonic pathogens are spread to patients during dental treatment.*

Introduction

Microorganisms from biological fluids may penetrate dental unit waterlines during patient treatment, particularly when the dental turbine hand-piece stops rotating (1). Indeed, oral streptococci, biological markers of saliva (2), are frequently found into dental unit water (DUW) after patient treatment with dental turbine (3) and human pathogens, such as Hepatitis C virus (4), Human Immunodeficiency virus (5), *Enterococcus faecalis* (6) and *Candida albicans* (7) are occasionally detected into DUW. The cross-infection risk due to these

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microorganisms is probably minimal, as no confirmed cases of infection are reported. Planktonic microorganisms detectable into DUW come also from the aquatic biofilm typical of oligotrophic environments (8) and opportunistic pathogens, such as *Pseudomonas aeruginosa*, *Legionella pneumophila* serogroup 1, non-tuberculous *Mycobacterium* spp. also are detectable into DUW (9, 10) and have been occasionally responsible for infections in susceptible individuals, such as elderly and cystic fibrosis patients (11-15).

The scientific evidence of high infection risk due to planktonic microorganisms transmitted through DUW among immunocompetent patients is lacking. Therefore, specific infection control measures are based on the Precautionary Principle, which states that when an activity presents an uncertain potential for substantial harm to human health, precautionary measures should be taken even if there is no scientific evidence that such measures are needed or effective (3). Water flushing from hand-pieces, use of anti-retraction devices and water disinfection (see (16-18) for review) are examples of Precautionary Principle-based infection control measures.

Disinfectants are generally, not necessarily, preferred to water flushing and anti-retraction devices due to valve deterioration and incomplete microorganism eradication by flushing (19, 20). Disinfectant activity is frequently tested against Heterotrophic Plate Count (HPC) bacteria (16) using laboratory and commercial kits (18, 21-23), because several scientific organizations (17, 18) suggested to adopt HPC-based quality standards of less than 500 (in the US) or less than 100 (in Europe) HPC colony forming units (cfu)/mL. These values followed the drinking water regulations established by the Environmental Protection Agency (EPA) (http://water.epa.gov/drink/contaminants/) and the European standard for bottled drinking water (http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:31998L0083&from=EN). However, although HPC-based methods are fast and cheap, their use to evaluate DUW quality is scientifically unsound. Indeed, the highest tolerable limit for HPC was proposed for drinking water by Allen and colleagues and the EPA, because elevated heterotrophic bacterial loads in water could interfere with the detection of total coliforms using the m-endo-based media, but Allen and the EPA also contended that *there was no scientific evidence that relatively high HPC loads are associable with presence of pathogens or pose per se an infectious risk* (24-26). Thus, in order to overcome the problem of testing DUW quality, the American Dental Association (ADA) Council on Scientific Affairs proposed to test dental unit water treatment systems using freshly isolated planktonic pathogens inoculated into DUW (27).

Hydrogen peroxide (H_2O_2)-monovalent silver ion (Ag^+) associations are acknowledged water treatments. They are extensively used for drinking water and wastewater treatments as chlorine supplements or substitutes, because their disinfection by-products are less toxic and mutagenic than chlorine by-products, are biodegradable and effective at the same time. Indeed, Ag^+ improves the oxidizing action of H_2O_2 by inducing the formation of hydroxyl radicals, that are strong oxidizing substances (28, 29). The effectiveness of H_2O_2-Ag^+ associations in DUW treatment resulted appreciable against HPC and aquatic microorganisms (30-34), but was not evaluated against human pathogens.

Thus, the aim of the present study was to evaluate the antimicrobial activity of H_2O_2-Ag^+-based disinfection against freshly isolated planktonic pathogens.

Materials and methods

This study was based on the guidelines of the European Committee for Standardization (CEN) to test: the fungicidal (35), bactericidal (36), mycobactericidal (37) activities of

chemical disinfectants for instruments used in the medical area; the anti-*Legionella* activity of chemical disinfectants for aqueous systems (38); the basic sporicidal activity (39). The methods adopted by the ADA Council on Scientific Affairs to evaluate DUW treatment systems (27) also were considered.

Tested microorganisms

Human pathogens potentially detectable into DUW after dental therapy, that is, *S. aureus*, *E. faecalis* and *C. albicans*, aquatic pathogens, that is, *P. aeruginosa*, *L. pneumophila* serogroup 1, and *M. chelonae*, and spores of *Bacillus clausii* were tested.

In accordance with ADA and in contrast with CEN, freshly isolated microorganisms were preferred to culture collection microorganisms, because the former are more resistant to antibiotics and disinfectants (40). Although detached parts of biofilm can be spread through DUW, there is no scientific evidence of infections due to sessile bacteria transmission in dental healthcare settings. For this reason, and in accordance with CEN and ADA methodologies, although bacteria organized in biofilm are more resistant to biocides (41), planktonic bacteria were tested.

S. aureus, *E. faecalis*, *C. albicans* and *P. aeruginosa* were isolated from patients with hospital-acquired infections and were kindly provided by the Microbiology Section of the Department of Public Health and Infectious Diseases of the Sapienza University (Rome, Italy). *M. chelonae* (42) and *L. pneumophila* (43) were isolated from the environment and were kindly provided by the Hygiene Section of the same Department. Commercially available spore suspensions of *B. clausii* were used. Microorganisms were stored at -20 C.

Experimental procedure

Before each testing occasion and for each strain, microorganisms, excluding spores, were subcultured using the appropriate media. Namely, Mannitol Salt Agar incubated 48 h at 37 C in aerobiosis for *S. aureus*; Enterococcosel Agar incubated 48 h at 37 C in aerobiosis for *E. faecalis*; Bismuth Sulphite Glucose Glycine Yeast Agar incubated 48 h at 30 C in aerobiosis for *C. albicans*; Pseudosel Agar incubated 48 h at 37 C in aerobiosis for *P. aeruginosa*; Middlebrook 7H10 incubated up to six weeks at 37 C in aerobiosis for *M. chelonae*; Charcoal-Yeast Extract Agar supplemented with Legionella BCYE-α Growth Supplement, incubated 10 days at 37 C in 2.5% CO_2 atmosphere for *L. pneumophila*. In order to obtain high bacterial loads, as suggested by CEN, five plates were inoculated for each strain, colonies were collected with a spatula and suspended into one tube containing 2 mL hard water (19.84 g $MgCl_2$, 46.24 g $CaCl_2$ in 1 L distilled water sterilized at 121 C for 15 minutes in autoclave –HW) and appropriate ten-fold dilutions in HW were performed.

The tubes were stored at 4 C and transported to the Department of Dental and Maxillofacial Sciences, where there was a dental unit equipped with a between-patient DUW disinfection system. At each testing occasion, 1 mL of a bacterial suspension was inoculated into the waterline of the test turbine, previously sterilized (121 C for 15 minutes in autoclave). The turbine was attached to the dental unit and submitted to the water treatment cycle. Namely, washing with sterile water, disinfection for 10 minutes with the H_2O_2-Ag^+-based disinfectant (H_2O_2 3% v/v, Ag^+ 0.001% w/v), washing to remove the residual disinfectant. At the end of the cycle the turbine was aseptically removed and immersed into a tube containing 10 mL HW and sodium thiosulfate (5 g/L), used to neutralize the residual disinfectant activity. This neutralizer was chosen because effective against both H_2O_2 and Ag^+ and because it has no antimicrobial activity (44). The remaining 1 mL of bacterial suspension was inoculated into the waterline of the

control turbine. The turbine was washed with sterile HW (5 mL) using a syringe to reproduce the washing phases of the disinfection cycle. Pilot tests performed with *S. aureus* and *P. aeruginosa*, showed that the microbial losses due to washing in the test and control turbines were similar and accounted for approximately 1 log cfu. The control turbine was left untreated for 10 minutes and was aseptically transferred into a tube containing 10 mL HW and sodium thiosulfate.

The dental unit was provided with sterilized HW and was not used for patient treatment throughout the period of the study. DUW disinfection was performed before the study start and the day before every testing occasion in order to decrease the number of bacteria potentially present in DUW which could interfere with the experimental procedures. Before the study start and 24 hours after the disinfection cycle, the contamination level of DUW was tested for HPC plating water samples and 1:10 dilutions on plates containing Bacto Yeast Extract Agar. One set of plates was incubated at 22 C and another set at 36 C for three days (45).

The tubes were stored at 4 C and transported to the laboratory, where they were processed within thirty minutes. They were vortexed for 5 minutes, then 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000 dilutions were made in HW. Aliquots of 0.2 mL of the undiluted suspension and of each dilution were plated in duplicate using the aforementioned media, specific for every strain (Tryptone Soy Agar was used for *B. clausii*) and incubated as previously described. The remaining undiluted suspension was filtered through a Millipore membrane (pore size, 0.45 μm), which was then plated and incubated. The use of specific selective media prevented the growth of other microorganisms, potential DUW contaminants, while for *B. clausii*, such a problem was overcome by counting only colonies with typical morphology.

Each strain was tested separately from the other strains for five times.

Statistical analysis

At every testing occasion, the microbial load detected with the control turbine was considered the pre-disinfection contamination level, the microbial load detected with the test turbine was considered the post-disinfection contamination level. Microbial loads were expressed in cfu.

For every species, median and range were used to express the central tendency of the five testing occasions and their variability, in order to account for the non-normal distribution of microbial loads (46). In order to assess the disinfectant activity, microbial loads were log transformed. When microorganisms were not detected, values were treated as 0.5, that is, one-half the distance between the lowest detectable level (i.e., 1 cfu) and zero. Mean log loads were calculated with 95% confidence intervals. According to CEN, the disinfectant activity was assessed as the reduction in number of log cfu (i.e., log reduction), which, for every test, was computed as the difference between the pre- and post-disinfection mean log loads.

In order to evaluate whether the H_2O_2-Ag^+-based disinfection was potentially able to eradicate the planktonic pathogens that can be detected into DUW, a literature search limited to the last 25 years was performed using PubMed and Scopus as databanks and "Dental Unit Water" as keywords. From the located studies, the highest water contamination levels of *S. aureus*, *E. faecalis*, *C. albicans*, *P. aeruginosa*, *L. pneumophila*, *M. chelonae* and *B. clausii* spores were searched and expressed as log cfu per water mL. If data about one species were not found, data regarding similar species were considered. The highest log loads which resulted from the literature search were compared with the log reductions obtained in the present

study. For every tested microorganism, if the log reduction was larger than the highest detectable log load it was assumed that the disinfectant activity was high enough to eradicate that microorganism from highly contaminated DUW.

Results

The total viable counts detected before the start of the study were 42 cfu/mL and 49 cfu/mL at 22 C and 36 C, respectively, low enough not to interfere with the tests. The physical characteristics of water were rather stable: temperature ranged between 18-22 C, residual chlorine between 0.08-0.25 mg/L, pH between 6.7-7.2 (data not in Table, kindly provided by the staff of the Unit of Environmental Hygiene of the Department of Public Health and Infectious Diseases and assessed during routine controls).

The median bacterial loads detected in the control turbines ranged between 800,000 (*L. pneumophila*) and 73,000,000 (*M. chelonae*) cfu (Table 1). The median loads detected in the test turbines were null for *S. aureus*, *E. faecalis*, *C. albicans* and *P. aeruginosa*, 90 cfu for *L. pneumophil*a, 465 cfu for *M. chelonae* and 32,100 cfu for spores.

The mean disinfectant activity of the H_2O_2-Ag^+ association exceeded 7 logs for *S. aureus*, *E. faecalis* and *P. aeruginosa*, 6 logs for *C. albicans*, 5 logs for *M. chelonae* and *L. pneumophila* and was almost 3 logs for *B. clausii* spores (Table 2).

The scientific literature search regarding DUW contamination provided 173 documents from Scopus and 151 from PubMed. The majority of these studies were not considered because reported quantitative data on HPC or qualitative data on specific microorganisms. The highest reported DUW contamination levels were the following: 14,995 cfu/mL for *Staphylococcus* spp. (47); 1,600 cfu/mL for *Enterococcus casseliflavus* (47); 66 cfu/mL for *Candida formata* (mean, 47 cfu/mL; standard deviations, 19 cfu/mL (48); 200,000 cfu/mL for *P. aeruginosa* (49); 8,300 cfu/mL for *L. pneumophila* (50); 2,056 cfu/mL for *M. chelonae* and *Mycobacterium gordonae* detected together in the same DUW sample (51); 1,144.86 cfu/mL for *Bacillus halodurans* (mean of 107 samples), it was not clear whether bacilli were actually spores or vegetative forms (52).

The log reductions attributable to H_2O_2-Ag^+-based disinfection were higher than the highest loads detectable into DUW for all the species excluding spores (Table 2).

Table 1 - Microbial loads (median, range) detected on the control and test turbines expressed in colony forming units (cfu).

Species	Control turbine		Test turbine	
	Median	Range	Median	Range
S. aureus	14,700,000	11,100,000-20,100,000	ND [a]	ND-ND
E. faecalis	20,100,000	9,300,000-24,300,000	ND [a]	ND-ND
C. albicans	948,000	564,000-1,470,000	ND [a]	ND-ND
P. aeruginosa	16,200,000	7,800,000-21,900,000	ND [a]	ND-ND
L. pneumophila	810,000	705,000-948,000	ND [a]	ND-90
M. chelonae	73,000,000	3,000,000-96,000,000	81	32-465
B. clausii (spores)	3,450,000	1,380,000-4,320,000	4,890	360-32,100

[a] not detected

Table 2 - Disinfectant activity of the H_2O_2-Ag^+-based formulation against the tested microorganisms (expressed as log cfu reduction, that is, the difference between mean log cfu detected in the control turbine and mean log cfu detected in the test turbine). Highest contamination levels reported by scientific literature for the tested (or similar) species, expressed in log cfu/mL.

Species	Log cfu reduction		Species	Highest Level
	Mean	95% Confidence Interval		
S. aureus	7.46	7.38-7.55	*Staphylococcus* spp.	4.18
E. faecalis	7.54	7.39-7.69	*Enterococcus casseliflavus*	3.20
C. albicans	6.28	6.13-6.44	*Candida formata*	1.82
P. aeruginosa	7.48	733-7.62	*P. aeruginosa*	5.30
L. pneumophila	5.35	4.33-6.38	*L. pneumophila*	3.92
M. chelonae	5.30	4.17-6.42	*M. chelonae*/*M. gordonae*	3.31
B. clausii (spores)	2.87	2.18-3.57	*Bacillus halodurans*	3.06

Discussion

The presence of biological fluids into DUW can be investigated using a surrogate marker of human saliva, that is, oral streptococci (2). Indeed, the habitat of oral streptococci is the human upper aero-digestive tract and all humans are colonized by these microorganisms at levels as high as 9 log cfu per saliva milliliter (53). The search of oral streptococci, however, may be problematic, because these microorganisms are difficult to cultivate when collected from the environment (6, 54). The search of surrogate markers instead of pathogens is the approach used to assess drinking water quality (24-26). The majority of surveys performed in dental healthcare settings found oral streptococci in DUW after dental treatment (3, 6, 8, 54-56), with few exceptions (34).

The frequent detection of oral streptococci in DUW suggests that patient-to-patient cross contamination is likely in routine practice. However, since there are no cases of cross infection, Precautionary Principle-based guidelines recommend to disinfect and/or purge DUW between patients (see, for example (17, 18, 57, 58).

The present study found that H_2O_2-Ag^+-based disinfection was active against planktonic pathogens coming from human biological fluids and those detached from aquatic biofilm, but did not investigate the antiviral activity. Indeed, HIV and HCV also have been detected into DUW (4,5). Therefore, it is important that disinfectants also are active against these microorganisms (59). Data extracted from different settings suggest that both H_2O_2 and Ag^+ are active against enveloped viruses, such as HIV, HCV, HBV (59). Nevertheless, it is impossible to infer that the tested disinfectant was active against pathogenic viruses into DUW.

The aim of this study was not to evaluate the effectiveness of H_2O_2-Ag^+-based disinfection against dental unit waterline biofilm or HPC, which was already and extensively investigated. Indeed, the antimicrobial activity against waterline biofilm, HPC and low loads of *P. aeruginosa* resulted moderate (30, 33, 34). The present study added further information regarding the activity of H_2O_2-Ag^+-based disinfectants and found an activity against human pathogens at contamination levels higher than those usually detectable into DUW.

Despite such disinfectant activity, several potential limits of between-patient H_2O_2-Ag^+-based disinfection of DUW, not evaluated in the present study, could come out in the long term. Indeed, frequent use of H_2O_2 at high concentration may deteriorate the dental equipment (31). In addition, the routine use of H_2O_2-Ag^+-based disinfectants could expose the dental staff to occupational risk of inhaling carcinogenic substances (60). Indeed, H_2O_2 by-products include several aldehydes and ketones, such as carcinogenic formaldehyde and acetaldehyde. Their production depends on both H_2O_2 concentration and quantity of organic material present in water (61). Finally, it is not possible to exclude that, like in waterline biofilm (62-64), H_2O_2 and Ag^+ may exert selective pressure on pathogens, thus promoting the development of resistant species.

In conclusion, the reported antimicrobial activity of H_2O_2-Ag^+-based disinfectant against the tested pathogens suggests that these products could be effective in controlling between-patient cross contamination. However, further studies are necessary to investigate whether the routine use of these substances may expose the dental staff to occupational risk.

Riassunto

Disinfezione con perossido di idrogeno e ioni argento monovalenti dell'acqua dei riuniti odontoiatrici contaminati artificialmente con microrganismi patogeni di recente isolamento

Obiettivi: L'acqua dei riuniti odontoiatrici può essere contaminata da patogeni provenienti da sangue e saliva di pazienti portatori e penetrati nelle tubature durante la terapia odontoiatrica, ovvero da patogeni opportunisti staccatisi dal biofilm che cresce sulle pareti delle tubature. Tali microorganismi possono essere trasmessi ai pazienti successivi. Abbiamo valutato l'attività disinfettante della combinazione perossido di idrogeno-ioni argento monovalenti (H_2O_2-Ag^+) nell'acqua di un riunito odontoiatrico contaminata artificialmente con microorganismi patogeni di recente isolamento.

Metodi: I microorganismi utilizzati (*Staphylococcus aureus*, *Enterococcus faecalis*, *Candida albicans*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Mycobacterium chelonae*, spore di *Bacillus clausii*) sono stati inoculati nelle tubature di turbine odontoiatriche precedentemente sterilizzate. Le turbine-test sono state connesse al riunito e trattate per 10 minuti con un disinfettante (H_2O_2 3%, Ag^+ 0.001%), le turbine-controllo non sono state trattate. Successivamente, le tubature delle turbine sono state lavate con acqua sterile usata poi per calcolare le cariche batteriche residue (espresse in unità formanti colonie –ufc). Ogni microorganismo è stato testato per cinque volte, per ognuno di essi sono state calcolate le medie logaritmiche. L'attività disinfettante è stata valutata in termini di abbattimento logaritmico medio (secondo European Standardization Committee), calcolato come differenza tra le cariche logaritmiche medie nelle turbine-controllo e nelle turbine-test.

Risultati: Le cariche batteriche medie nelle turbine-controllo sono risultate comprese tra 10^5-10^7 ufc. Gli abbattimenti logaritmici medi sono stati: 7.5 log ufc per *S. aureus*, *E. faecalis*, *P. aeruginosa*, 6.3 per *C. albicans*, 5.4 per *L. pneumophila*, 5.3 per *M. chelonae*, 2.9 per le spore di *B. clausii*.

Conclusioni: La disinfezione dell'acqua dei riuniti con la combinazione H_2O_2-Ag^+ può ridurre il rischio di trasmissione di microorganismi patogeni di provenienza umana o di patogeni opportunisti dell'acqua ai pazienti in corso di trattamento odontoiatrico.

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