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Original Research Article

Evaluation of the level of microbial contamination and prevalence of gram-negative non-fermentative rods in dental unit waterlines

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Abstract

Introduction: The cross infection control in dental office has received great attention from professionals and one of the critical points is the bacteriological control of water used in dental unit. **Objective:** To perform a microbiological evaluation of the water used in dental units, the identification of Gram-negative non-fermentative rods (GNNR) and their ability to adhere to polystyrene, and the antimicrobial activity of disinfectants on the identified strains. **Material and methods:** The heterotrophic bacteria count and GNNR identification were performed on water samples collected from 25 dental units (air/water syringe and reservoir). The GNNR were assessed on their capability to adhere to polystyrene and on their antimicrobial activity to the following disinfectants: sodium hypochlorite (0.06%, 0.12%, 0.25%, and

0.5%) and chlorhexidine (0.03%, 0.06%, and 0.12%). **Results:** 88% of the air/water syringe collected samples and 68% of the reservoir collected samples were out of the potability standards. The quantity of isolated bacteria from the reservoir was lower than from the air/water syringe in 88% of the dental units. *Methylobacterium* spp. was found in highest percentage (19.7%) during GNNR genus isolation. There was a weak adherence to polystyrene in 85.04% of the samples. Sodium hypochlorite at 0.25%, inactivated 100% of the GNNRs in 10 minutes, while the highest tested concentration of chlorhexidine (0.12%), inactivated 98.5% of the GNNRs. **Conclusion:** These results provide information on the contamination problem of dental unit waterlines (DUWL) and indicate a need for treatment of the water used in dental units. The disinfection of DUWL can be performed with sodium hypochlorite at 0.25% (half the concentration recommended in the literature). However, further studies are necessary regarding DUWL frequency disinfection.

Introduction

Despite the efforts to avoid cross-infection in dental office using sterilized instruments, individual protection equipment and disinfection procedures, other measures such as microbiological control of the water used in dental units are required to prevent the spread of diseases [10, 11].

The quality of water in dental units is of considerable importance because both patients and dental staff are regularly exposed to water and aerosol generated by these units [20, 23].

A single dental chair unit can be used in the treatment of many patients each day, and microbial contamination of specific component parts can be a significant potential source of cross-infection [10].

There are currently no official standards or legislation regarding microbial quality of dental unit waterlines (DUWL). Furthermore, until recently, there has been hardly any specific guidance from dental chair unit manufacturers on dental chair unit supply water quality [5]. Actually, the responsibility for ensuring that dental chair units provide good quality water output has, by and large, been considered to rest on the shoulders of dental practitioners and/or dental clinic management.

The literature reports the use of several methods to reduce or eliminate this bacterial contamination [1, 16, 21]. Yet, there is no standard procedure and the water used in dental units during dental treatment still shows high amounts of heterotrophic bacteria [25]. This contamination can be originated from the suction of microorganisms from the patient's mouth or derive from the multiplication of microorganisms contained in the water supply or in the biofilms present in DUWL [18, 23, 26].

The Brazilian Health Ministry Resolution n. 518 [4] states that for water to be considered safe for human consumption, it should contain a maximum of 500 colony forming units per milliliter (CFU/ml), given it is free of coliforms. Both the Center for Control and Prevention of Diseases (CCD) and the American Dental Association (ADA) recommend the use of sterile water on surgical procedures with bone exposure [2], and that the water used on non-invasive procedures do not exceed 200 CFU/ml.

Dental units are equipped with a network of small bore semi-rigid plastic tubes (two to three mm) which provide water to the air/water syringe and to the handpieces [3]. The water used comes from reservoirs coupled to the unit directly from tap water. Different studies show that water coming from dental units can be contaminated with microorganisms such as *Pseudomonas* spp., *Acinetobacter* spp., *Burkolderia* spp., *Alcaligenes* spp., *Methylobacterium* spp., *Sphingomonas* spp., *Flavobacterium* spp., and *Moraxella* spp., favoring biofilm formation on DUWL [1, 18, 28]. The bacteria on the biofilm are adhered to a surface and produce extracellular polymers that ease adhesion and are even more protected from the action of antimicrobials, bacteriophages, phagocytic amoebas and from desiccation [18].

Several authors have suggested the use of disinfectants for decontamination of DUWL [1, 18, 21], however there is no agreement on which product is the most effective.

Considering the possible contamination of dental units due to colonization by microorganisms capable of forming biofilm, the concerns were to evaluate the bacteriological quality of water used in dental units (air/water syringe and reservoir) through the total count of heterotrophic bacteria; to isolate and identify Gram-negative nonfermentative rods (GNNR) present in water; to verify the adherence capability to polystyrene and the antimicrobial activity of different sodium hypochlorite (0.06%, 0.12%, 0.25%, 0.5%) and chlorhexidine (0.03%, 0.06%, 0.12%) concentrations against the isolated bacteria.

Material and methods

Collection of water samples

Water samples were collected from 25 dental units, being 15 from the Universities Dental Clinics (units 1 – 15); four from the Community Welfare University Center (units 16 – 19); six from the Dental Association (units 20 – 25). All samples were collected from air/water syringe and reservoir of each dental unit.

Decontamination was performed preceding collection on the external surface of the air/water syringe and reservoir through cloth friction with 70% (v/v) alcohol. A 20 to 30 seconds continuous flush was purged prior to water collection from the air/water syringe, simulating the recommended procedures for the use of equipment [6]. The reservoirs were disconnected from the units for water collection, and in order to neutralize the residual chlorine from chlorine-treated water samples, approximately 100 ml of water was collected from the air/water syringe and from the reservoirs in previously sterilized flasks containing 0.1 ml 10% (w/v) sodium thiosulfate solution (Reagen, Brazil).

Total heterotrophic bacteria count

The samples were homogenized and diluted 1:10 and 1:100 in 0.9% (w/v) physiologic solution. One hundred microliters of the pure samples and dilutions were uniformly applied on the surface of plate count agar (PCA) (Difco, USA). The plates were incubated at 30ºC for 48-96 hours. The reading was carried out on the plates which showed between 30 and 300 CFU after the 48 and 96 hours incubation period. The experiments were performed in duplicate.

To evaluate whether there was a statistically significant difference regarding the contamination level of the syringe and reservoir, the Qui-square test with a 0.01 significance level was applied.

Isolation and identification of Gram-negative nonfermentative rods (GNNR)

The morphologically different colonies isolated on the PCA plates were submitted to the Gram stain. The Gram-negative bacteria were identified according to the technique described in the Manual of Clinical Microbiology [17] and through the kit NF-Prov (nonfermenters) (Newprov, Paraná, Brazil).

Adhesion to inert surface (polystyrene)

Adhesion to inert surface was assessed by employing the method described by Stepanovic *et al.* [22] with some modification. The GNNR were incubated for 24 – 48 hours at 30ºC in Tryptic Soy Broth (TSB) (Difco, USA). The cultures were diluted 1:200 in TSB, and 200 μL of this suspension was inoculated in quadruplicate in sterile 96-well polystyrene plates (NUNC, Naperville, IL) and incubated for 24 hours at 30ºC, while negative control wells contained broth only. Then, the content of each well was aspirated and the wells were washed three times with 250 μL phosphate-buffered saline (PBS-pH 7.2). The attached bacteria were fixed with 200 μL methanol p.a (Merck, Germany) per well, and after 15 minutes the plates were emptied and left to dry. The plates were stained for five minutes with 0.2 ml 2% (w/v) Hucker crystal violet per well. Excess stain was rinsed off by placing the plate under running tap water. The plates were air-dried and the optical density (O.D.) of each well was measured at 550 nm with a Micro-ELISA Autoreader (MultiScan EX, Labsystem, Uniscience).

The cut-off O.D. (O.Dc) was defined as three standard deviations above the mean O.D. of the negative control. Strains were classified as follows: non-adherent (O.D. $\leq$ O.Dc), weakly adherent (O.Dc $<$ O.D. $\leq$ 2 x O.Dc), moderately adherent (2 x O.Dc $<$ O.D. $\leq$ 4 x O.Dc) and strongly adherent (4 x O.Dc $<$ O.D.).

Evaluation of disinfectants antimicrobial activity

Sodium hypochlorite solutions of different concentrations were used: 0.06% (600 p.p.m.), 0.12% (1200 p.p.m.), 0.25% (2500 p.p.m.) and 0.5% (5000 p.p.m.). Chlorhexidine solutions were used in the following concentrations: 0.03%, 0.06%, and 0.12%.

The assay was performed in duplicate, with some modifications, and according to the technique described by Litsky and Litsky [12]. The GNNR strains were inoculated in TSB and incubated for 24 – 48 hours at 30ºC.

After the incubation period, these cultures were standardized according to the turbidity, with tube one of the McFarland scale to obtain a suspension with 10^8 microorganisms/ml, and 1 ml of this suspension was then added to 4 ml disinfectants. The tubes were manually agitated for 1 minute and left at room temperature for 10 minutes. One hundred microliters of this mixture was uniformly applied on the surface of PCA containing neutralizer and incubated for 48 – 96 hours at 30ºC with colony counts performed after incubation. The assay performed with sodium hypochlorite had a 0.6% (w/v) sodium thiosulfate (Reagen, Brazil) neutralizer, and 0.5% (w/v) Tween 80 and 0.07% (w/v) soy lecithin (Sigma, USA) with chlorhexidine.

Results

Table I shows the heterotrophic bacteria counts found in the water collected from the reservoirs and from air/water syringes of the 25 dental units. The obtained count average in the water samples from the reservoirs was of $4.0 \times 10^4 \pm 1.0 \times 10^5$ ranging from 2.0×10^1 to 4.6×10^5 CFU/ml. The count values in the air/water syringes ranged from 1.1×10^2 to 4.6×10^5 CFU/ml, and the obtained average was $8.4 \times 10^4 \pm 1.1 \times 10^5$. According to the obtained averages of the heterotrophic bacteria count, there was a statistically significant difference regarding the contamination level of the water collected from the air/water syringe and from the reservoir when the Qui-square test with $\alpha < 0.01$ was used.

Table I – Determination of the number of heterotrophic bacteria in water samples from reservoirs and air/water syringes collected from 25 dental units

Unit	Total heterotrophic bacteria count (CFU/ml)*	
	Reservoir	Air/water syringe
01	$2,0 \times 10^2$	$2,5 \times 10^4$
02	$4,3 \times 10^3$	$8,1 \times 10^4$
03	$1,9 \times 10^4$	$2,2 \times 10^5$
04	$1,0 \times 10^3$	$1,9 \times 10^5$
05	$3,3 \times 10^3$	$3,7 \times 10^4$
06	$8,0 \times 10^2$	$7,5 \times 10^3$
07	$1,4 \times 10^2$	$8,0 \times 10^3$
08	$1,8 \times 10^2$	$7,9 \times 10^3$
09	$1,8 \times 10^2$	$1,7 \times 10^4$
10	$7,0 \times 10^2$	$2,9 \times 10^5$
11	$7,0 \times 10^2$	$3,6 \times 10^4$
12	$4,6 \times 10^5$	$4,2 \times 10^4$
13	$8,0 \times 10^3$	$6,7 \times 10^4$
14	$1,4 \times 10^5$	$1,9 \times 10^5$
15	$2,0 \times 10^2$	$1,8 \times 10^5$
16	$1,4 \times 10^5$	$4,6 \times 10^5$
17	$4,1 \times 10^4$	$7,3 \times 10^4$
18	$1,1 \times 10^4$	$1,7 \times 10^4$
19	$1,7 \times 10^5$	$1,3 \times 10^5$
20	$2,0 \times 10^1$	$2,5 \times 10^2$
21	$1,0 \times 10^3$	$1,1 \times 10^2$
22	$1,5 \times 10^2$	$4,0 \times 10^2$
23	$3,4 \times 10^2$	$5,4 \times 10^2$
24	$5,8 \times 10^2$	$7,9 \times 10^3$
25	$1,6 \times 10^3$	$8,0 \times 10^2$
average ± S.D.†	$4,0 \times 10^4 \pm 1,0 \times 10^5$	$8,4 \times 10^4 \pm 1,1 \times 10^5$

* Count average. Tests performed in duplicate

† Standard deviation

The number of isolated bacteria from the reservoir was lower than the isolated samples from the air/water syringes in most units (except for numbers 12, 19, 21, and 25).

According to the Brazilian Health Ministry Resolution n. 518 [4], 88% (22/25) of the water samples from the air/water syringes and 68% (17/25) of the samples from reservoirs showed results above potability bacterial standards (table I).

GNNR isolated from air/water syringe and reservoir water samples are shown on table II. *Methylobacterium* spp. was the highest percentage isolated genus, (19.7%), followed by *Moraxella* spp. (15.2%) and *Acinetobacter* spp. (13.6%). Microorganisms belonging to the same genus were recovered in the reservoir and air/water syringe in 24% of dental units.

Table II – Gram-negative nonfermentative rods isolated from water samples from reservoirs and air/water dental units syringes

Unit	Reservoir	Air/water syringe
01	*Acinetobacter calcoaceticus* *Stenothrophomonas maltophilia*	*Delftia acidovorans* 01 GNNR*
02	01 strain not recovered†	*Moraxella osloensis* 01 GNNR
03	*Stenothrophomonas maltophilia*	*Stenothrophomonas maltophilia* 01 GNNR
04	*Burkholderia cepacea*	*Burkholderia cepacea* *Sphingomonas paucimobilis*
05	*Acinetobacter* spp. *Stenothrophomonas maltophilia*	*Methylobacterium* spp. *Sphingomonas paucimobilis* *Alcaligenes faecalis*
06	01 strain not recovered	*Moraxella osloensis* *Methylobacterium* spp. *Acinetobacter haemoliticus*
07	*Methylobacterium* spp. *Moraxella osloensis*	*Methylobacterium* spp. 01 GNNR
08	*Sphingomonas paucimobilis* *Moraxella catarrhalis*	*Sphingomonas paucimobilis* *Alcaligenes faecalis*
09	01 strain not recovered	*Moraxella osloensis*
10	*Methylobacterium* spp.	*Sphingomonas paucimobilis*
11	*Delftia acidovorans* *Methylobacterium* spp.	01 strain not recovered
12	*Methylobacterium* spp. *Delftia acidovorans* *Acinetobacter iwoffii* *Sphingomonas paucimobilis*	01 strain not recovered
13	01 strain not recovered	*Moraxella osloensis* *Acinetobacter iwoffii*
14	*Alcaligenes faecalis* *Moraxella catarrhalis*	*Methylobacterium* spp. *Moraxella osloensis*
15	*Sphingomonas paucimobilis* *Methylobacterium* spp.	*Moraxella catarrhalis*
16	01 strain not recovered	*Pseudomonas stutzeri* *Acinetobacter calcoaceticus*
17	*Methylobacterium* spp.	01 strain not recovered
18	*Pseudomonas stutzeri* *Flavobacterium mizutaii* *Alcaligenes faecalis*	*Pseudomonas stutzeri* *Acinetobacter baumannii*
19	*Pseudomonas stutzeri* *Acinetobacter calcoaceticus* 01 GNNR	*Delftia acidovorans* *Methylobacterium* spp. 01 GNNR

Table II (continuation)

Unit	Reservoir	Air/water syringe
20	01 strain not recovered	*Acinetobacter haemoliticus*
21	02 strains not recovered	02 strains not recovered
22	*Methylobacterium* spp.	*Burkolderia cepacea*
23	02 samples not recovered	*Moraxella osloensis*
24	01 strain not recovered†	01 strain not recovered
25	*Methylobacterium* spp.	*Alcaligenes faecalis*

* Gram-negative nonfermentative rods not identified
† Strains not recovered in the adopted standards

Of the studied strains 85.04% (57/67) showed weak adherence to polystyrene. Only one showed strong adherence and seven showed moderate adherence.

Sodium hypochlorite at 0.06% concentration inactivated 56.1% of the strains and 89.4% at 0.12%. All strains were inactivated at 0.25% (table III). 72.7% of the tested strains were inactivated with chlorhexidine at 0.03%, 90.9% at 0.06%, and 98.5% at 0.12% (table III).

Table III – Effect of different sodium hypochlorite and chlorhexidine concentrations on Gram-negative nonfermentative rods (GNNR) isolated from water from 25 dental unit water lines

Isolated bacteria (n)	Inactivation by sodium hypochlorite			Inactivation by chlorhexidine		
	0.06% n (%)	0.12% n (%)	0.25% n (%)	0.03% n (%)	0.06% n (%)	0.12% n (%)
Methylobacterium spp. (13)	8/13 (62%)	11/13 (85%)	13/13 (100%)	9/13 (69%)	12/13 (92%)	12/13 (92%)
Moraxella spp. (10)	8/10 (80%)	8/10 (80%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)
Acinetobacter spp. (9)	6/9 (67%)	9/9 (100%)	9/9 (100%)	6/9 (66.6%)	7/9 (78%)	9/9 (100%)
Sphingomonas paucimobilis (7)	4/7 (57%)	6/7 (86%)	7/7 (100%)	5/7 (71%)	7/7 (100%)	7/7 (100%)
GNNR not identified (6)	3/6 (50%)	5/6 (83%)	6/6 (100%)	4/6 (67%)	5/6 (83%)	6/6 (100%)
Alcaligenes faecalis (5)	3/5 (60%)	4/5 (80%)	5/5 (100%)	4/5 (80%)	5/5 (100%)	5/5 (100%)
Pseudomonas stutzeri (4)	1/4 (25%)	4/4 (100%)	4/4 (100%)	3/4 (75%)	4/4 (100%)	4/4 (100%)
Stenothrofomonas maltophilia (4)	1/4 (25%)	2/4 (50%)	4/4 (100%)	3/4 (75%)	4/4 (100%)	4/4 (100%)
Delftia acidovorans (4)	1/4 (25%)	4/4 (100%)	4/4 (100%)	2/4 (50%)	2/4 (50%)	4/4 (100%)
Burkolderia cepacea (3)	1/3 (33%)	3/3 (100%)	3/3 (100%)	1/3 (33%)	3/3 (100%)	3/3 (100%)
Flavobacterium mizutaii (1)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)
Total (66)	**37/66 (56%)**	**59/66 (89%)**	**66/66 (100%)**	**48/66 (73%)**	**60/66 (91%)**	**65/66 (98%)**

Discussion

If the water used in dental unit reservoirs remains with high microbial contamination rates it will continue to be an infection source to the dentistry practice [13, 28]. Most DUWL microorganisms do not represent a risk to public health, but are considered opportunistic microorganisms, and therefore, liable to cause diseases to immunosuppressed patients [23].

DUWL may create favorable conditions for biofilm formation due to their small bore, wall tube imperfections, water flow, presence of minerals and organic molecules, and frequent rest periods [2, 18]. Under these conditions, the microorganisms present in the water used to supply the dental units multiply and form biofilm on the luminal tube surfaces [18, 24].

The number of isolated bacteria in water samples from air/water syringes is usually higher than the ones isolated from reservoirs, once with the water flow through the dental unit lines there is the release of bacteria belonging to the biofilm [18, 26]. Hence, even when using water of good origin, if biofilm is present in DUWL the water ejected through the air/water syringe will be contaminated with bacteria belonging to the biofilm [18].

In our study, four units showed lower CFU in air/water syringe in comparison to the reservoir. This variation may be attributed to the heterogeneous distribution of bacteria in the water sample or to the age of the unit. When new, they may not show

biofilm yet, and therefore, not show such different results in the water from the air/water syringe and reservoir. The same count behavior can occur if the unit is old but the lines have recently been replaced.

Variation in the total microorganism count can occur even when using appropriate culture media, temperature and incubation time since bacteria have slow growth, and some are not cultivable in the used mediums and conditions, and others are not recovered [24, 25, 27]. These reasons, together with the difficult identification of GNNR explain why many microorganisms were isolated from the reservoir but not from the syringe and vice-versa.

The reason heterotrophic bacteria number readings were performed in 48 and 96 hours in our study is because in 48 hours we had the result of the total number of heterotrophic bacteria, but colony pigmentation only occurred after 96 hours of incubation.

We set 30ºC as the incubation temperature for our experiments since several papers reported that at this temperature there had been greater recovery of microorganisms [3, 27].

This paper focused on the identification and study of GNNR. Studies performed in different countries have reported the prevalence of GNNR, opportunistic and adapted to water which proliferate and form biofilms [3, 18, 28]. Despite the possible DUWL contamination with microorganisms from the patients' mouths, oral bacteria are not usually present in dental unit waters due to the use of antiretraction valves and sterile handpieces, which control the suction of these microorganisms [26].

Bacteria found in our study were predominantly environmental organisms. Some of the bacteria identified (*S. maltophilia*, *B. cepacea*, *P. stutzeri*, *Acinetobacter* spp.) are known as opportunistic pathogens.

Martin [13] reported two cases of infections caused by *P. aeruginosa* acquired by immunocompromised patients after restorative dentistry treatment. In Spain, Fernández-Cuenca *et al.* [7] performed a survey of GNNR associated with hospital infection and found that the most important were: *S. maltophilia*, *A. baumannii* and *P. aeruginosa*, and Kawamura *et al.* [9] in Japan reported a case of recurrent bacteremia associated with venous catheter in an 11 years old girl, associated with *D. acidorovans*.

The results of adhesion to polystyrene showed that most of the samples showed weak adherence. This is probably due to the kind of bacteria studied, since they showed a slow growth. The results could have shown a greater number of moderately or weakly adhering bacteria if other methodologies or a longer incubation period had been used. Another factor that might have contributed to the increased number of bacteria with weak adherence was the non-addition of glucose to the culture medium (recommended in most techniques), once we strongly sought to match the conditions found in dental unit water.

The most efficient means of achieving good quality DUWL water output is through regular treatment∨disinfection of DUWL with a chemical, biocide or cleaning agent that removes biofilm from DUWL effectively, and therefore, resulting in good quality water output [5, 19].

Sodium hypochlorite and chlorhexidine were tested because they are products frequently used in dentistry. Chlorine compounds have been studied more extensively than any other class of chemical agents intended to control or eliminate biofilm in DUWL [1, 8]. Several studies indicate the use of sodium hypochlorite at 0.5% (5000 p.p.m.) to disinfect DUWLs [8, 21]. However, due to its high corrosive power, we tested smaller concentrations for the same purpose.

Sodium hypochlorite at 0.25% (2500 p.p.m.) was able to inactivate all tested microorganisms. We suggested the use of sodium hypochlorite at 0.25% to decontaminate DUWL, due to possible damages, both to patients and professionals and also to equipment, from the use of a more concentrated solution. However, more studies are necessary about the frequency of DUWL decontamination with sodium hypochlorite at 0.25% and its impact on equipment, on other microorganism groups (Gram-positive bacteria, *Mycobacterium* spp., fungi) and on the removal of already-present biofilm in the DUWLs.

The most resistant microorganisms to sodium hypochlorite at 600 p.p.m. were the *P. stutzeri* and bacteria that belonged to *Pseudomonas* genus, that is, *Burkolderia* spp., *D. acidovorans* and *S. maltophilia*. While *S. maltophilia* was the microorganism that showed the highest number of strains resistant to chlorine at 1200 p.p.m. concentration. Due to their ability to survive in aqueous mediums, these microorganisms became particularly problematic in hospital environments, once it is frequently associated with hospital infections [7, 9].

Chlorhexidine is probably the most used compound in oral anti-septic compositions. New products with this active principle have been recommended by the FDA (Food and Drug Administration) to control DUWL contamination [15]. Chlorhexidine shows good disinfectant activity and wide action range, nevertheless, there are disadvantages such as possible skin irritability, high costs, possibility of color changes in restorations, teeth and tongue and taste modification when continuously used [14].

Conclusion

According to our results, this paper indicates a need for treatment of the water used in dental units and provides information on the contamination problem of DUWL. The decontamination of DUWL can be performed with sodium hypochlorite at 0.25% (half the concentration recommended in the literature). However, further studies are necessary regarding DUWL frequency decontamination.

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