

RESEARCH ARTICLE

Enrichment Cultures should be performed in the detection of Bacterial Oral Human Pathogens in DUWLs

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Abstract

Water delivered by dental units during routine dental practice is densely contaminated by bacteria. The aim of this study was to determine actual isolation of the microorganisms sprayed from Dental Unit Water Lines (DUWLs) when enrichment cultures are performed and to compare frequencies with those obtained without enrichment cultures. Moreover, the antimicrobial susceptibilities of the microorganisms isolated were also studied. Water samples were collected from one hundred dental equipments in use at Dental Hospital of our University in order to evaluate the presence/absence of microorganisms and to perform their presumptive identification. Aliquots from all of the samples were inoculated in eight different media including both enrichment and selective media. Minimal inhibitory concentrations (MIC) were determined by the broth dilution method. The results herein reported demonstrate that most of the DUWLs were colonized by bacteria from human oral cavity; when enrichment procedures were applied the percentage of DUWLs with detectable human bacteria was one hundred percent. The results showed that in order to evaluate the actual risk of infections spread by DUWLs the inclusion of a step of pre-enrichment should be performed. The need for devices preventing bacterial contamination of DUWLs is a goal to be achieved in the near future that would contribute to maintain safety in dental medical assistance.

Key words: Bacterial colonization, DUWLs, enrichment cultures.

Introduction

The persistent presence of microorganisms in dental unit water lines (DUWLs) is well

Documented^{1,2,3}. A large number of investigations dealing with different strategies to control the eventual infections spread through these devices has been carried out in the last few years⁴. Numerous species of pathogen and non-pathogen microorganisms enter dental units retracted up from the oral cavity of patients undergoing dental treatment or delivered by incoming municipal water. The microorganisms isolated from samples obtained from DUWLs include, in principle, bacteria and fungi able to originate biofilm in the inner surface of the DUWLs. The well-established biofilm constitutes a reservoir of bacteria; in most cases these bacteria are saprophytic, heterotrophic, Gram-negative, and aerobic or facultative. These bacteria are currently considered as non-pathogenic in dentistry. However, the complexity of bacterial communities living in DUWLs is remarkable and comprises almost 40 genera including β and γ proteobacteria such as *Afipia*, *Sphingomonas*, *Pseudomonas*, *Leptospira*, *Bacillus*, *Escherichia*, *Geobacter*, *Legionella* and others^{5,6}. The presence of bacteria such as *Pseudomonas aeruginosa* cannot be considered as harmful since on compromised patients they can originate a waste variety of severe infections. Moreover, some authors have proposed DUWLs as a potential vehicle contributing significantly to spread oral infectious agents between patients and/or between patients and dentists. If this is so, these microorganisms should be sucked into the rotary instruments during one treatment and expelled during the next⁷. In fact, the American Dental Association (ADA) has established a goal for dental water to contain no more than 200 Colony Forming Units (CFU)/ml of heterotrophic bacteria.

This paper is devoted to isolation, quantitative determination and presumptive identification

of microorganisms emerging from DUWLs at Dental Hospital of our University during regular use of the dental equipments. Moreover, antimicrobial susceptibility of the potential pathogen strains was also tested in order to evaluate the ability of these organisms to acquire antibiotic resistance.

Materials and methods

Media and chemicals

Anaerobic agar, *Veillonella* agar, *Mitis salivarius* agar and R2A agar were used to isolate oral microorganism. All these media were purchased from Difco (Detroit, Michigan, USA). Trypticase Soy Agar (TSA), Columbia blood agar base and Mueller Hinton Broth (MH) (which was used to determine antimicrobial susceptibility) were purchased from Scharlau Bacteriology Products (Spain). Finally *Actinomyces* Selective medium (CFAT) was prepared in our lab with the following composition: Trypticase Soy broth (30 g/L); Dextrose (5 g/L); Cadmium sulphate (13 mg/L); Sodium fluoride (80 mg/L); acriflavine (1,2 mg/L); potassium telurite (2.5 mg/L); basic fuchsin (0.25 mg/L) and Bactoagar (15 g/L). After sterilisation at 121°C, 50 ml/L of horse defibrinated blood were added. Anaerobic enrichment cultures were performed by using double concentrated thioglycolate broth (Scharlau, Barcelona, Spain). Fungi were counted in Sabouraud-dextrose agar supplemented with 25 mg/L of chloramphenicol (Scharlau, Barcelona, Spain).

Ciprofloxacin was kindly supplied by CENAVISA Laboratories (Reus, Spain) and the rest of antibiotics and chlorhexidine were purchased from SIGMA (Sigma Aldrich Corporation, St. Louis, Missouri, USA). Finally, TAED (TetraAcetylEthyleneDiamine) peroxidant and Benzidamin HCl were kindly supplied by Castellini SpA and Farma Lepori respectively (both from Italy), Triclosan (Ciba *especialidades químicas* S.L. Spain)

Sample collection and growth conditions

The dental Clinic of the University is equipped by one hundred dental equipments. These equipments were purchased from different suppliers including all European highly prestigious manufacturers. Regularly the equipments are in use from several years ago (less than 10) along ten hours/day and either by professors of dentistry and Graduate students. Units are connected with municipal water supply. Samples of 10 ml of water were collected in disposable sterile tubes and immediately transported to the laboratory

(less than 12 minutes between collection and inoculation of primary media). All of these samples were inoculated in the previously mentioned different media.

Cultivable heterotrophic bacteria were determined on R2A agar by inoculation of 1ml of sample per plate and incubated at 37°C during 4 days in anaerobic conditions, a replica was incubated aerobically. Total count in aerobic conditions was also made on Columbia blood agar and Trypticase Soy agar (TSA) with incubation at 37°C aerobically for 24 hours. Total anaerobic bacterial population was determined by inoculating plates of Anaerobe Agar containing 5% defibrinated horse blood. *Streptococcus* involved in oral infections was determined by the use of Mitis-Salivarius Agar incubated at 37°C in microaerophilic conditions (5% CO₂ in a Heareus autozero Incubator) for 4 days. Microorganisms of the genus *Veillonella* were counted by the use of *Veillonella* agar, this medium contains basic fuchsin and vancomycin and is selective for the isolation of *Veillonella*, plates were also incubated at 37°C in anaerobic conditions for 4 days. Finally a selective medium for isolation of *Actinomyces viscosus* and *A. naeslundii* was inoculated and incubated for 5 days in anaerobic conditions⁸.

All of the plates were analyzed visually, colonies counted and morphological characteristics of the colonies described. Pure cultures from all different morphologically distinguishable colonies were prepared for further studies. The different isolates were submitted to Gram staining and observed under the microscope (Jenamed, Carl Zeiss Jena Germany); when necessary biochemical tests were carried out.

Plates to count fungi were incubated aerobically at 27°C for three weeks, plates were examined, colonies described and fungi identified *de visu*.

The second step of this work was a repetition of the mentioned methods preceded by an enrichment step. Samples were, in these cases, used to inoculate a tube containing 10 ml of thioglycolate broth (double concentrated). Tubes were incubated for 24 hours at 37°C and enrichment culture used to inoculate the different media as reported above.

Antimicrobial susceptibility test

Minimal inhibitory concentrations (MIC) were determined by the broth dilution method. Overnight cultures of the bacterial strains in Mueller-Hinton broth (with supplements when necessary) were diluted 100-fold in fresh broth and inoculated into Mueller-Hinton broth

containing serial dilutions of the antimicrobial agents (inocula size was approximately 0.5×10^4 cfu/ml). MICs were determined after 18 h or 36 h (depending on species) of incubation at 37°C as the minimum concentration of antibiotic that inhibits growth.

Results

Isolation and characterization of the oral human bacteria from the DUWLs

Columbia blood agar as well as on TSA gave in most cases high numbers of bacteria within a wide range of counts (from less than 100 ufc/ml to more than 5×10^5 ufc/ml) (Figure 1).

Average of number of heterotrophic bacteria was of 2.1×10^5 ufc/ml with a standard deviation as high as 2.9×10^4 . Figure 2 shows percentages of the samples colonized by heterotrophic bacteria.

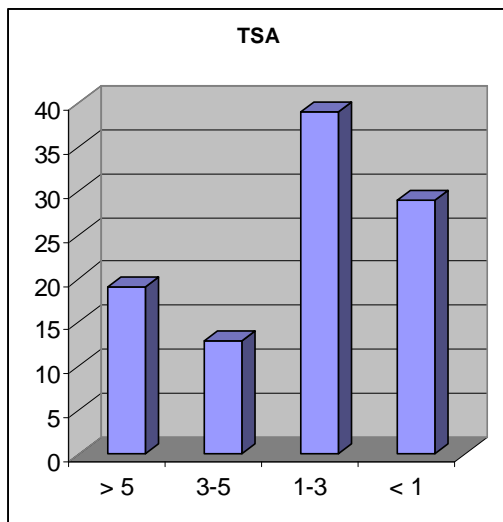
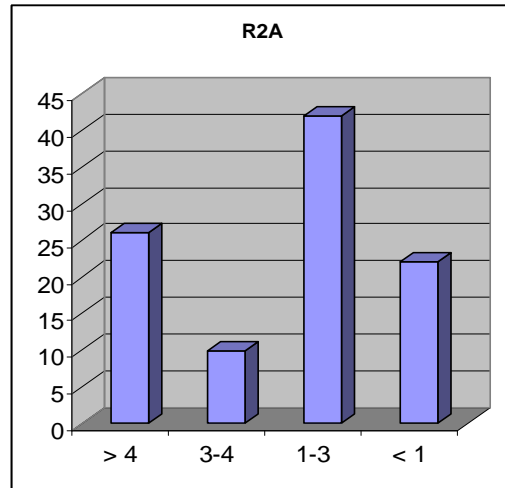
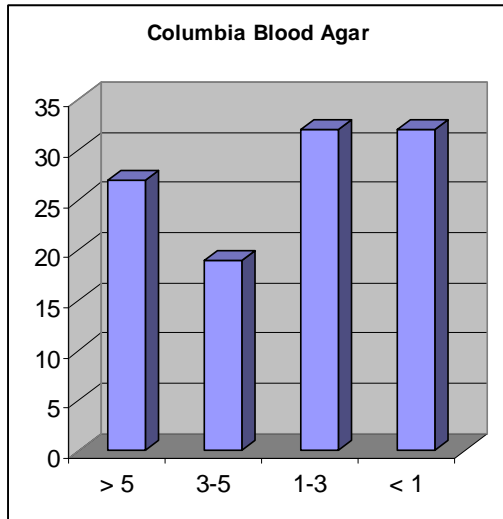


Fig. 2 Percentage of samples (DUWL's) colonized by bacteria cultivable on R2A. (Logarithm of colony forming units/ml)

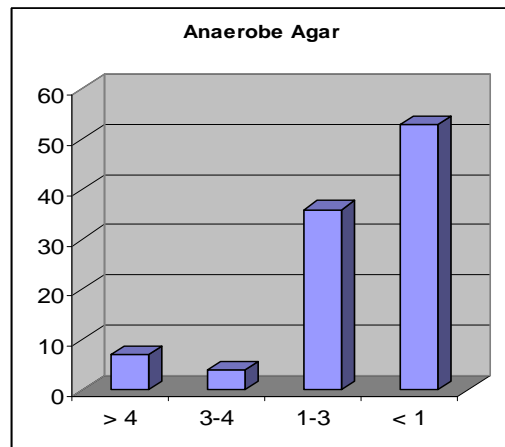


Fig. 1 Percentage of samples (DUWL's) colonized by bacteria cultivable on columbia blood agar and TSA. (Logarithm of colony forming units/ml)

Fig. 3 Percentage of samples (DUWL's) colonized by bacteria cultivable on Anarobe agar (Logarithm of colony forming units/ml)

First of all it should be noted that even when no enrichment step was applied, 97% of the DUWLs studied resulted to be colonized by different kinds of bacteria. Counts on

Total anaerobic bacteria counted onto Anaerobic agar were much lower than aerobic and facultative bacteria (Figure 3), ranging from 0 to $2 \cdot 10^4$ ufc/ml. Anaerobic bacteria were detected in 45 DUWLs among the 100 scored. *Veillonella* was detected in 24 DUWLs among the 100 scored using non-enrichment procedures and in all cases the number of colonies was lower than 40 ufc/ml. Comparison of bacterial growth either with or without enrichment is showed in Figure 4. When the presence of *Streptococcus mitis* and *S. salivarius* was studied, less than 13 % of the DUWLs studied resulted to be positive without enrichment. On the contrary 83 % of DUWLs contained detectable *S. mitis* or *S.*

salivarius after enrichment. Finally the detection of *Actinomyces viscosus* and *A. naeslundii* gave similar results than in the case of streptococci, since only 58 % of DUWLs were positive without enrichment and all equipments tested were positive after enrichment. It should be noted that *Pseudomonas* species was isolated from 35 of the equipments studied.

Fungal presence in DUWLs was detected in 34% of tested equipments. Fungal species were *Fusarium spp* (19%); *Acremonium spp.* (11%), *Penicillium spp.* (2%) and *Candida albicans* (2%).

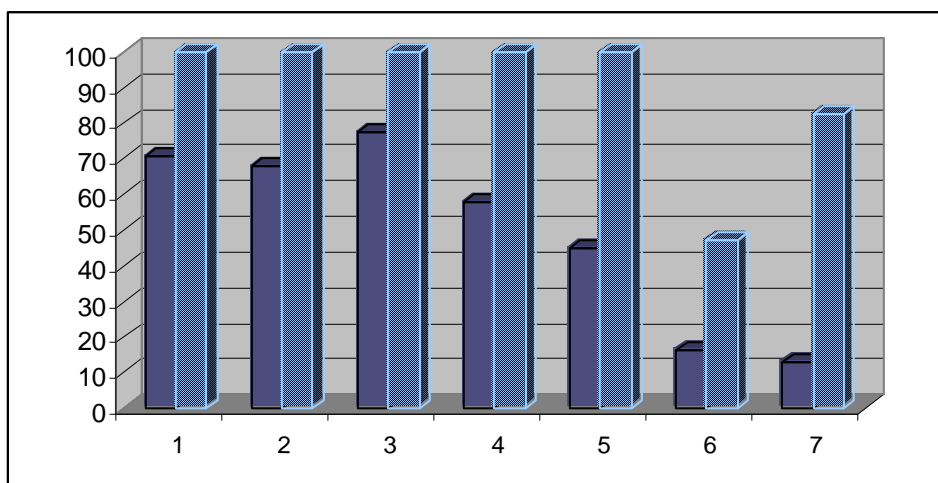


Fig 4. Bacterial growth on the different used bacteriological media (X-axis) either with or without enrichment (1. TSA; 2. Columbia blood agar ;3. R2A; 4. *Actinomyces* agar ; 5. Anaerobe agar; 6. *Veillonella* agar; 7. Mitis-Salivarius agar)

Antimicrobial susceptibility

Tables 1 and 2 summarize the results of antimicrobial susceptibility of the bacterial isolates. Antimicrobials tested included both antibiotic and disinfectants commonly used in dentistry.

Discussion

The presence of adherent microbial biofilms in dental waterlines has been demonstrated by different authors^{9,10}. It has been shown that DUWLs are densely colonized during routine dental practice by heterotrophic bacteria mostly coming from municipal water supplying dental units or sucked¹¹. In some cases, human pathogens are sucked back into the lines during dental

procedures due to inappropriate work of anti-retraction mechanisms included in dental units. The limited effectiveness of anti-retraction valves to prevent cross-contamination has been clearly demonstrated¹².

The interest in the study of DUWLs stable biofilms and the eventual colonization of DUWLs by human oral bacteria is mainly due to the concern originated by the increasing number of immunocompromised patients as well as the emerging awareness of occupational hazards in the dental offices⁹.

The results herein reported demonstrate that most of the DUWLs are colonized by bacteria. These results evidence the existence of an intern contamination in the waterlines of the dental units and also show

STRAINS	TAED	Benzidamine	Chlorhexidine	Triclosan
<i>Veillonella spp.</i>	230	>750	0.1	0.7
<i>Bacillus spp.</i>	940	>750	0.2	1.4
<i>Bacillus subtilis</i>	940	750	0.2	1.4
<i>Streptococcus spp.</i>	470	>750	0.2	93.7
<i>Capnocytophaga spp.</i>	230	750	0.1	5.8
<i>Stomatococcus spp.</i>	940	750	0.2	1.4
<i>Actinobacillus spp.</i>	230	750	0.05	0.7
<i>Actinomyces spp.</i>	230	750	0.1	0.7

TABLE 1. Minimal inhibitory concentrations (MICs) in µg/ml of different chemical agents to oral microorganism isolated. *TAED (TetraAcetylEthyleneDiamine) peroxidant

the possibility of a “cross contamination” between patients and patients and dentists. Moreover, we tried to detect the actual presence of the so-called oral pathogens in the DUWLs. By direct enumeration it was possible to demonstrate the presence of such bacteria in a relatively high proportion of DUWLs studied (figure 1). When microbiological enrichment procedures were applied the percentage of positive DUWLs increased dramatically. In general it is assumed that human pathogens cannot multiply in external media and that when these microorganisms are in the environment, they can occasionally survive, although they do it in a stressed state. Moreover, several authors have pointed out that it is feasible to detect the presence of bacteria still alive but unable to form colonies on conventional culture media. These microorganisms so called VBNC

(Viable but non cultivable) can be active as infectious agents but, in general, microbiologists cannot detect them by conventional methods. Among cultivable pathogenic bacteria surviving in cold and, theoretically, clean water such as that of the DUWLs it is feasible that most of them are in low proportion when compared with bacteria forming biofilms. Additionally, they should be stressed; subsequently the viability by direct plating on selective media is highly limited. However, when a pre-enrichment step in a rich medium such as thioglycolate was done, all microorganisms were allowed to grow and subsequently the number of almost any kind of bacteria able to multiply in such a medium increased rapidly. When these cultures were used to inoculate selective media the successful detection of human pathogens

STRAINS	Ery	Mtz	Cf	Cip	Str	Amp	Va	Tet	Cli
<i>Veillonella spp.</i>	0.63	<0.078	0.63	0.63	1.56	6.25	6.25	0.78	>25
<i>Bacillus spp.</i>	<0.078	<0.078	0.16	0.31	1.56	<0.078	3.12	0.78	3.12
<i>B.subtilis</i>	0.31	<0.078	0.16	0.31	>3.12	<0.078	<0.078	1.56	>25
<i>Streptococcus spp.</i>	<0.078	<0.078	0.63	0.16	1.56	6.25	1.56	0.78	12.5
<i>Capnocytophaga spp.</i>	<0.078	<0.078	0.63	0.16	>3.12	3.12	1.56	0.78	6.25
<i>Stomatococcus spp.</i>	<0.078	<0.078	0.63	0.16	>3.12	3.12	<0.078	0.78	>25
<i>Actinobacillus spp.</i>	0.16	0.16	0.16	0.31	>3.12	3.12	1.56	0.78	25
<i>Actinomyces spp.</i>	<0.078	<0.078	<0.078	0.16	3.12	1.56	<0.078	0.78	25

TABLE 1. Minimal inhibitory concentrations (MICs) in µg/ml of different antimicrobial agents and chemical agents to oral microorganisms isolated (Ery: Erythromycin; Mtz: Metronidazole; Cf: Cephalothin; Cip: Ciprofloxacin; Str: Streptomycin; Amp: Ampicillin; Va: Vancomycin; Tet: Tetracycline; Cli: Clindamycin)

increased drastically as can be seen in figure 4. Thus, it is clear that in order to evaluate the actual risk of infections spread by DUWLs the inclusion of a step of pre-enrichment is highly recommended.

The presence of opportunistic pathogens in DUWLs should also be taken into account. We were able to determine the presence of *Pseudomonas aeruginosa* in a large proportion of DUWLs. In principle *P. aeruginosa* is considered as an opportunistic pathogen highly virulent in some particular conditions such as immunocompromised patients either AIDS patients or immunosuppressed individuals. Most of these patients require dental assistance since one of the clinical manifestations of their disease is periodontitis, in these cases exceptional caution should be exercised in order to prevent occasional infections^{13,14}.

Despite fungal presence seemed not to be relevant in comparison with bacterial presence (34 in front of 100% colonization), results also suggest that the main source of colonization should be water supply, since *Candida* was isolated only in 2% of equipments.

The active presence of either pathogenic and non-pathogenic bacteria in DUWLs and the eventual failure of retraction valves to prevent the entry of oral bacteria into the water lines, makes necessary to explore mechanisms or strategies in order to sterilize or disinfect the inner part of DUWLs between patients. This should include the design of devices improving the disinfection from all points of view as well as their adaptation into dental equipments. Concerning antimicrobial susceptibility, the results of the MICs showed great differences between chemical agents such as TAED, Chlorhexidine, Triclosan or Benzidamine HCl, and antibiotics (Tables 1 and 2). Isolates resulted to be susceptible to chemical agents. TAED-peroxidant¹⁵ exhibited good activity against all bacterial isolates. Despite the high incidence of multiresistant bacteria in bacterial populations in Spain is a well known phenomenon, we failed in the isolation of resistant bacteria to the antibiotics tested. This is in principle a positive result in terms of public health, since antibiotic resistance is becoming an increasing public health concern. Moreover our results clearly pointed out that no multiresistant bacteria could be isolate from DUWLs. This could be due to the low incidence of multi-drug

resistant bacteria in mouth, which would be unexpected in a community with high incidence of multiresistance. Further research to determine the index of occurrence of multi-drug resistant bacteria in mouth should be done.

Acknowledgments

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