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BACTERIAL DECONTAMINATION OF DUWL BIOFILM USING OXYGENAL 6

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Abstract: The aim of this study was bacteriological assessment of the dental unit waterlines (DUWL) biofilm - concentration and composition of the aerobe and facultative anaerobe bacterial microflora, and evaluation of the influence of a disinfecting product, Oxygenal 6, on the biofilm composition. Tubing fragments were taken from 25 units twice, before and after disinfection, and bacterial suspension of the biofilm was obtained from the samples. The bacterial flora was determined with the plate culture method. Bacteria were identified with biochemical microtests: API 20E, API 20NE (bioMérieux, France) and GP2 MicroPlate™ (BIOLOG, USA). Before disinfection, the following bacteria were identified: Gram-negative bacteria - *Ralstonia pickettii*, *Pseudomonas vesicularis*, *Sphingomonas paucimobilis*, *Xanthomonas maltophilia*; Gram-positive cocci - *Micrococcus luteus*, *Micrococcus lylae*, *Staphylococcus cohnii*, *Staphylococcus lentus*, *Staphylococcus* spp., *Streptococcus* spp.; Actinomycetes - *Streptomyces albus*. The prevailing bacteria were: *Ralstonia pickettii* (78.62%), found in all the units, and *Sphingomonas paucimobilis* (20.45%). After DUWL disinfection, *Sphingomonas paucimobilis* (88.79%) dominated in the biofilm, *Staphylococcus* spp. - 5.61% and *Pseudomonas* spp. - 3.74% were next most frequently occurring bacteria, and in more than a half of the biofilm samples 100% reduction of the bacterial microflora occurred. This study confirms effectiveness of Oxygenal 6 in bacterial decontamination of the DUWL biofilm.

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Biofilm, which forms on the wall of dental unit waterlines, is considered as a source of microbiological water contamination [7, 11, 12]. Therefore, it seems justified that disinfection, the aim of which is to guarantee a good microbiological quality of water, should be targeted at the biofilm, and evaluation of a disinfectant should be based on its effectiveness against the biofilm in the conditions of a dental surgery [17].

The aim of this study was bacteriological assessment of the DUWL biofilm - concentration and qualitative composition of aerobe and facultative anaerobe of the bacterial microflora, and evaluation of the influence of a disinfecting product, Oxygenal 6, on the biofilm composition.

MATERIAL AND METHODS

The study included 25 dental units located in public dental clinics. To bacteriologically assess the biofilm formed on the inside walls of the unit waterlines, a 15 mm-long fragment of the tubing was aseptically taken from each unit. The tube fragments were taken twice: before disinfection, and on the 15th day after implementing a disinfecting procedure. The tube fragments were immersed in 2 ml of sterile buffered solution of physiological salt with calcium chloride and magnesium chloride, and shaken in order to obtain bacterial suspension.

Concentration and qualitative composition of bacterial microflora in the supernatant liquid were determined with

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the plate dilution method using surface culture on the appropriate agar media: blood agar to determine the total number of bacteria and mesophilic actinomycetes, eosine methylene blue (EMB) agar to identify Gram-negative rods. Tenfold dilutions with the sterile solution of physiological salt were prepared from the initial water samples. Next, 0.1 ml of the examined liquid was taken from each dilution and evenly spread on the surface of the agar media in 2 parallel repetitions. The cultures on blood agar and EMB agar were incubated for 24 hours at 37ºC, 3 days at room temperature (22ºC), and 3 days at 4ºC. Prolonged incubation at lower temperatures was to enable growth of some meso- and psychrophilic species.

After incubation, preliminary identification of bacteria grown on each medium was performed following instructions of the manuals (Bergey's Manual…) [5, 9, 19]: the colonies grown were first assessed macroscopically, considering such characteristics as size, shape, structure, colony colour, etc., and next with microscopic methods, staining the bacterial preparations with Gram method. The total number of bacteria and the number of particular morphological types were determined, and their concentration was reported as colony forming units in 1 ml of water - cfu/ml.

Next, the strains of most frequently occurring bacteria were isolated and identified to the level of species or genus with biochemical microtests: API 20E test (bioMérieux, Marcy l'Etoile, France), which is used to identify Gram-negative bacteria of Enterobacteriaceae family and other fermenting Gram-negative rods, API 20NE test (bioMérieux, Marcy l'Etoile, France) to identify non-fermenting Gram-negative rods, and GP2 MicroPlate™ test (BIOLOG, Inc., Hayward, CA, USA) used to determine Gram-positive bacteria. All the tests were performed according to the manufacturer's instructions.

API 20E and API 20NE test technique. An appropriate API system to identify Gram-negative rods was selected after testing the ability of producing cytochrome oxidase by the examined strains. To establish this, the bacterial mass was applied to the reacting surface of a test strip (Bactident Oxidase, Merck, KGaA, Germany) and after 20-60 seconds results were read. Oxidase-positive strains, causing the strip to change colour to blue or purple-blue, were identified with API 20NE test, while oxidase-negative strains (causing no colour change) were determined with API 20E test. Biochemical identification systems, API 20E and API 20NE, consisting of 20 microtubes, containing dehydrated substrates, were filled with bacterial suspension of an appropriate opacity in sterile physiological salt, and subsequently incubated for 18-24 hours at 35ºC (API 20E) and 24-48 hours at 30ºC (API 20NE). Metabolic processes occurring during incubation caused changes in colour which followed spontaneously or after application of indicatory reagents. After reading the reaction, strain identification was obtained on the basis of a 7-digit numerical profile, found in API 20E or API 20NE code books.

GP2 MikroPlate™ test technique. GP2 MicroPlate™ is a standardised micromethod to identify Gram-positive bacteria on the basis of their metabolic pattern, using 95 biochemical characteristics. BIOLOG system determines the ability of microorganisms to use or to oxidise compounds which are carbon sources. The examined strains of Gram-positive bacteria were suspended to a specified density in 0.4% gelifying solution, and after adding 3 drops of sodium tioglycolate (5 mM) which inhibits false positive responses, 150 µl of the suspension was pipetted into each of 96 wells of the plate. The microplates were incubated for 24 hours at 35ºC. The colour indicator used in the test - tetrazolium violet - responded to the metabolic processes by changing colour into purple in the wells positive for a given strain. The results were read after 6 and 24 hours of incubation, comparing the colour of liquid in the wells with the reference negative control. The results were subsequently analysed with MicroLog™ computer programme, provided by the manufacturer. To identify a given bacterial species with the programme, the similarity index match (SIM) should reach at least 0.75 for the microplates read after 6 hours of incubation, and at least 0.50 for the microplates incubated for 24 hours.

Application of a disinfecting procedure. The used disinfectant was Oxygenal 6 (KaVo, Germany) containing 6% hydrogen peroxide whose action is enhanced by silver ions. The product is designed for the use in dental surgeries and admitted to trading in Poland. The disinfectant was used according to the manufacturer's instructions to obtain a desired hydrogen peroxide concentrations. DUWL disinfection procedure was two-stage. First, DUWL underwent intensive disinfection with 0.25% hydrogen which was kept present in all the waterlines elements for 30 minutes thanks to a continual flow of water from the reservoir to the handpieces - water was flushed through all the unit handpieces. The second stage consisted in the constant presence of 0.02% hydrogen peroxide in DUWL for 2 weeks.

Statistical analysis. The obtained results were processed using Microsoft Excel 2000, Statistica 5.1. The assumed error risk was 5%.

RESULTS

The identified bacteria were found in all the biofilm samples taken from the inside walls of dental unit waterlines. Before disinfection, the following Gram-negative bacteria were present: *Ralstonia pickettii* (*Pseudomonas pickettii*), *Pseudomonas vesicularis*, *Sphingomonas paucimobilis*, *Xanthomonas maltophilia*; Gram-positive cocci: *Micrococcus luteus*, *Micrococcus lylae*, *Staphylococcus cohnii*, *Staphylococcus lentus*, *Staphylococcus* spp., *Streptococcus* spp. and actinomycetes: *Streptomyces albus*. *Ralstonia pickettii* were present at all operative sites. *Sphingomonas paucimobilis* was isolated from the biofilm taken at 12 sites,

Table 1. Bacteria identified in the biofilm at individual operative sites before disinfection and after disinfection.

	Site number	
Bacteria	Before disinfection	After disinfection
Gram-negative bacteria		
Ralstonia pickettii (Pseudomonas pickettii)	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25	
Pseudomonas diminuta		9
Pseudomonas vesicularis	9, 18	
Sphingomonas paucimobilis	1, 2, 3, 4, 6, 9, 14, 16, 18, 19, 21, 25	1, 2, 3, 9, 13, 14, 16, 21
Xanthomonas maltophilia	13, 14, 15, 16, 21, 25	
Gram-positive bacteria		
Cocci:		
Micrococcus luteus	2, 4, 5, 8, 9, 15, 18	11, 22
Micrococcus lylae	6	
Staphylococcus cohnii	5, 7, 8, 9, 12	
Staphylococcus lentus	14, 16	
Staphylococcus spp.	6	1, 4, 15, 16, 21
Streptococcus spp.	6	
Actinomycetes:		
Streptomyces albus	4	

Table 2. Average concentration (cfu/ml) and proportion of particular genera/species of bacteria in biofilm samples before disinfection and after disinfection.

Bacteria of genus/species	Before disinfection		After disinfection	
	cfu/ml	%	cfu/ml	%
Pseudomonas spp.	186,233.2	78.81	1.6	3.74
Sphingomonas paucimobilis	48,319.2	20.45	38.0	88.79
Staphylococcus spp.	213.6	0.09	2.4	5.61
Streptococcus spp.	16.0	0.01	0.0	0.00
Micrococcus spp.	215.2	0.09	0.8	1.87
Xantomonas maltophilia	1,318.4	0.56	0.0	0.00
Streptomyces albus	0.4	0.0002	0.0	0.00
Total	236,316.0	100	42.8	100

Micrococcus luteus - at 7 sites, *Xanthomonas maltophilia* - at 6 sites, *Staphylococcus cohnii* - at 5 sites (Tab. 1).

In the biofilm, before disinfection the average concentration of total bacteria in all the samples was 236,316 cfu/ml, the minimum - 4,840 cfu/ml, and the maximum - 756,000 cfu/ml (Tab. 2, 3).

Ralstonia pickettii (*Pseudomonas pickettii*) prevailed, constituting 78.61% of the total isolated bacteria. *Sphingomonas paucimobilis* (20.45%) and *Xanthomonas maltophilia* (0.56%) were second most frequently found bacteria (Tab. 2).

Ralstonia pickettii (*Pseudomonas pickettii*) - aerobe, Gram-negative, non-fermenting, oxidase-positive rods, may be isolated from environmental and clinical samples. They are believed to be of little clinical importance; the literature, however, reports numerous cases of infection with this microorganism, especially in hosts with immunity impaired as a result of an underlying disease [5, 8, 18]. *Sphingomonas paucimobilis* - aerobe Gram-negative rods, may be isolated from different environments and human-related sources; they are associated with infections connected with the use of catheters [14].

Table 3. Concentration of bacteria isolated from the biofilm before and after DUWL disinfection (cfu/ml).

	Before disinfection			After disinfection		
	Mean	Min	Max	Mean	Min	Max
Gram-negative bacteria	235,870.8	4,300	756,000	39.6	0	330
Gram-positive cocci	444.8	0	3,000	3.2	0	20
Actinomycetes	0.4	0	10	0	0	0
Micrococcus bacteria	215.2	0	2,000	0.8	0	10
Pseudomonas pickettii	185,777.2	2,430	752,000	0	0	0
Staphylococcus bacteria	213.6	0	2,000	2.4	0	20
Total bacteria	236,316	4,840	756,000	42.8	0	330

mean - mean concentration for all sites, min - minimum concentration at a site, max - maximum concentration at a site.

After application of a disinfecting procedure, both the number of isolated bacterial species and their concentration, decreased. Bacteria isolated from 12 biofilm samples, *Sphingomonas paucimobilis* - from 8 samples, *Staphylococcus* spp. - from 5 samples, *Micrococcus luteus* - from 2 samples, *Pseudomonas diminuta* - from one sample (Tab. 1).

After DUWL disinfection, *Sphingomonas paucimobilis* (88.79%) dominated in the biofilm, *Staphylococcus* spp. - 5.61% and *Pseudomonas* spp. - 3.74% were next most frequently occurring bacteria (Tab. 2). The average concentration of total bacteria was 42.8 cfu/ml. Bacteria were not found in all the biofilm samples. The maximum concentration of bacteria in the biofilm at one operative site was 330 cfu/ml (Tab. 2, 3).

The Wilcoxon test for related variables was used to compare bacterial concentration in the biofilm before and after application of a disinfecting procedure. A hypothesis that there is no difference between bacteria concentration before and after DUWL disinfection, was verified with this test.

Table 4. Statistical analysis of DUWL disinfection influence on bacterial concentration in the biofilm (Wilcoxon test).

Bacteria	T	Significance	p
Gram-negative bacteria	0.00	***	0.000012
Gram-positive cocci	12.00	**	0.0038
Micrococcus bacteria	3.00	*	0.012520
Staphylococcus bacteria	6.00	*	0.016374
Pseudomonas bacteria	0.00	***	0.000012
Total identified bacteria	0.00	***	0.000012

T - Wilcoxon test value for groups: n 25; p - significance level for Wilcoxon test; * - p<0.05, ** - p<0.01, *** - p<0.001.

Table 5. Number of genera/species of bacteria identified in the biofilm before and after DUWL disinfection.

Site	Before disinfection	After disinfection
1	2	2
2	3	1
3	2	1
4	4	1
5	3	0
6	5	0
7	2	0
8	3	0
9	5	2
10	1	0
11	1	1
12	2	0
13	2	1
14	4	1
15	3	1
16	4	2
17	1	0
18	4	0
19	2	0
20	1	0
21	3	2
22	1	1
23	1	0
24	1	0
25	3	0

Tabela 6. Analysis of influence of DUWL disinfection on the number of genera/species of bacteria isolated from the biofilm.

Bacteria	T	Significance	p
Biofilm	0.00	***	0.000040

T - Wilcoxon test value for groups n 25; p - significance level for Wilcoxon test, *** - p<0.001.

For the needs of statistical analysis, the following groups were distinguished: Gram-negative bacteria, Gram-positive cocci, bacteria of *Micrococcus* genus, bacteria of *Staphylococcus* genus, bacteria of *Pseudomonas* genus, total identified bacteria.

The statistical analysis showed a highly significant decrease in the concentration of Gram-negative bacteria, *Pseudomonas* bacteria and total identified bacteria in the biofilm after using a DUWL disinfectant. A decrease in the concentration of Gram-positive cocci, *Micrococcus* and *Staphylococcus* bacteria was statistically significant (Tab. 4). In the biofilm, after DUWL disinfection, *Streptococcus* bacteria and actinomycetes were not found (Tab. 1). This confirms the claim concerning the disinfectant effectiveness.

Wilcoxon test was used to analyse a decrease in the number of genera/species of bacteria isolated from the biofilm samples taken after application of a disinfecting procedure. The analysis showed that the decrease was highly statistically significant (Tab. 5, 6).

DISCUSSION

Control methods of the microbial quality of DUWL water include different DWUL treatment methods of varying effectiveness [12].

Studies concerning the treatment of dental water and its quality have focused on two principal issues: reducing the microbial count of water samples to or below the 200 cfu/ml standard recommended by the ADA in 1964, and identifying effective mechanical techniques and chemical disinfectants for treating dental unit waterlines [4].

According to Walker and Marsh [17], many researchers and dentists were, and still are, primarily concerned only with the bacteria present in the water-born phase. This is understandable as it is only the liquid, and hence the bacteria in the liquid-phase, with which the patient comes in contact. However, these bacteria are often derived from biofilm community that establishes on the tubing. Consequently, the properties and significance of heterogeneous mass of bacteria that attach and grow on the inside wall of the piping within DUWL as a biofilm have not always been addressed.

Therefore, its seems essential to investigate the influence of disinfectants on the DUWL biofilm.

The finding by Walker *et al.* [15] that there was a direct correlation between the numbers of bacteria in biofilm and planctonic samples, confirms the need to focus DUWL water control activities on biofilm. However, it should be remembered that the DUWL biofilm control is difficult due to biofilm specific properties [17].

The biofilm bacteria usually exist in a microniche, in a complex microbiological community which is characterised by a primitive homeostasis, circulation systems, and metabolic cooperation. Bacteria established in biofilm show different responses than the same cells in the planctonic form [1, 2, 3, 7, 10].

According to the accepted hypotheses, the resistance of biofilm to antimicrobial products is due to: 1. slow, incomplete or faulty penetration of antibacterial substances through the biofilm matrix; 2. changed growth rate of biofilm forming organisms; 3. other physiological changes resulting from modifications of biofilm growth, caused mainly by the conditions existing in the microenvironment. Extracellular polymeric substances which form the biofilm matrix, constitute a diffusive barrier for preparations and drugs, slowing down the rate of particles transport to deep layers of the biofilm structure, or through a specific reaction of a drug with the matrix polymer.

It was found that metabolism, multiplication of microorganisms, and cell growth in biofilm are considerably slower than in analogical organisms in the planctonic form. It is probably related to a limited availability of nutrients and oxygen to the biofilm cells, and a significant decrease in the growth rate, or even bacteria entering a resting state, contribute to reducing sensitivity of microorganisms in biofilm. Steward and Costerton [10] claim that a slower growth rate of microorganisms in biofilm constitutes a physiologically distinct form of bacterial life, a specific phenotype which is conditioned genetically.

According to the third hypothesis, the same conditions which negatively influence the activity of antimicrobial products in vitro, found in the biofilm microenvironment, are responsible for its resistance to drugs.

Analysing effectiveness of disinfectants, the properties of the biofilm-forming bacteria should be considered - their known resistance to disinfecting products and antibiotics, which has been described in the literature [10].

It should be underlined that after disinfection with Oxgenal 6, in more than a half of the samples 100% reduction of the aerobe and facultative anaerobe bacterial microflora in biofilm occurred (Tab. 5). The obtained results are worse than those by Walker *et al.* [16] who, examining the influence of various disinfecting products on the microbiological quality of biofilm, achieved 100% reduction in the biofilm total viable counts (TVC) and a >95% reduction in biofilm coverage where Oxygenal was used. An unquestionable asset of the mentioned research was the use of an established biofilm laboratory model, while present study was conducted in the conditions of a general dental practice.

It should be stressed that the fact that the disinfectant significantly reduces concentrations of Gram-negative bacteria that are the main source of endotoxin - an important factor in inflammations - is an advantage [13].

In this study, the microbial quality of the DUWL biofilm samples taken in the conditions of a general dental practice was adopted as a criterion of decontaminating effectiveness of the evaluated product. The study confirmed effectiveness of Oxygenal 6 in clinical practice as a bacterial decontamination product for the DUWL biofilm.

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