Full Paper

Susceptibility of bacteria isolated from dental unit waterlines to disinfecting chemical agents

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Susceptibility testing of bacteria to disinfecting chemical agents isolated from dental unit waterlines (DUWL) is necessary for the development of effective disinfectant products. However, until now, susceptibility tests for chemical agents, which are components of DUWL disinfectant products, have not been conducted on bacteria isolated from DUWL water. The aim of this study was to evaluate and compare the susceptibilities of DUWL isolates in planktonic and biofilm states to cetylpyridinium chloride, as well as to the four chemical agents currently used for DUWL management. A total of 56 isolates, including 12 genera, were identified by 16S rDNA sequencing, and one strain of each genus was selected for susceptibility testing. A total of 12 isolates were used for the susceptibility tests. We determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the planktonic state and the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) for the biofilm state using microtiter plates. MIC, MBC, MBIC, and MBEC of the 12 isolates for ethanol were the highest, followed by sodium hypochlorite, hydrogen peroxide, and chlorhexidine. Similar to chlorhexidine, the lowest MIC, MBC, MBIC, and MBEC were found in cetylpyridinium chloride. The susceptibilities of the isolates for sodium hypochlorite and ethanol were similar in the planktonic and biofilm states. For hydrogen peroxide and chlorhexidine, the MBIC and MIC were similar, but MBEC was 256 times higher than MBC. The MBIC and MBEC of isolates for cetylpyridinium chloride were 128 and 256 times higher than the MIC and MBC, respectively. As far as we know, this was the first study reporting the susceptibility of DUWL isolates to cetylpyridinium chloride and chemical agents used for disinfecting DUWLs. Cetylpyridinium chloride, for which the DUWL isolates showed the highest susceptibility, could be used for disinfecting DUWLs.

Key Words: biofilm; cetylpyridinium chloride; dental infection control; disinfectants; susceptibility

Introduction

The Centers for Disease Control recommends that the levels of bacteria in the water discharged from dental units should not exceed 500 colony forming units per mL (CFU/mL) and the American Dental Association recommends not exceeding the more stringent 200 CFU/mL (ADA, 1999; Kohn et al., 2004). However, previous studies have shown that bacterial contamination levels in the dental unit waterlines (DUWL) exceed recommended levels (Barbeau et al., 1996; Walker et al., 2004; Yoon and Lee, 2015). There are many studies that have confirmed contamination levels in DUWL water. Furthermore, some studies have also identified species of bacteria present in DUWL water (Costa et al., 2015; Singh et al., 2003). Currently, chemical agents used for disinfecting DUWLs include chlorine dioxide, sodium hypochlorite, hydrogen peroxide, and chlorhexidine gluconate. In previous studies, the disinfectant products were applied to the dental unit and their efficacies were confirmed by comparing the total number of bacteria and the biofilm accumulation in the DUWLs before and after the disinfection (Kettering...
et al., 2002; Schel et al., 2006). However, efficacy tests of disinfectant products for individual bacteria have not yet been conducted. The disinfectant products containing sodium hypochlorite or hydrogen peroxide, which showed the best efficacy, were reported to have side effects such as corrosion of dental equipment, clogging of the DUWLs, and stimulation of the oral mucosa (Lin et al., 2011; O’Donnell et al., 2011; Schel et al., 2006). Therefore, it is necessary to develop disinfectant products that are low cost and have few side effects on dental units and the oral cavity. Susceptibility testing of bacteria to antimicrobials provides information on the minimum concentrations of chemical agents that can remove bacteria, and this information can be useful for the development of efficient disinfectant products (Meiller et al., 2001). However, until now, the susceptibility test for the chemical agents used for disinfecting DUWLs against individual bacteria isolated from DUWL has not been conducted.

Cetylpyridinium chloride is commonly used as an antimicrobial compound and is classified as a safe and effective compound in the United States (Pandit et al., 2015; Witt et al., 2005). The positively charged cetylpyridinium chloride has antimicrobial activity that damages cells by binding to the surface of negatively charged bacteria and interacting with the bacterial cell membrane (Sreenivasan et al., 2013; Sutherland, 1985). With safety, cetylpyridinium chloride has antimicrobial activity against various microorganisms including oral bacteria. In some studies, cetylpyridinium chloride has been shown to also have anti-biofilm activity (Ioannou et al., 2007; Pandit et al., 2015; Ramalingam et al., 2012).

The purpose of this study was to determine the susceptibilities of DUWL isolates in planktonic and biofilm states to chemical agents used for disinfecting DUWLs and to compare the susceptibilities of DUWL isolates to cetylpyridinium chloride as a new possible chemical agent for disinfecting DUWL.

Materials and Methods

Sampling. Fifteen dental units supplied with water from the main tank connected to a chlorinated municipal water supply were used in the experiments to isolate bacteria. Five hundred milliliters of water samples were collected in a sterile glass bottle from an ultrasonic scaler of each dental unit. In order to prevent external contamination, the outlet and external surfaces of the DUWLs were cleaned with 70% ethanol before the collection. To neutralize residual chlorine in the collected water, 0.5 mL of 10% sodium thiosulfate (Yakuri Pure Chemicals Co., Ltd, Kyoto, Japan) solution was added to each sample. These samples were immediately transferred to the laboratory.

Detection of bacteria and pure culture. Water samples collected from each dental unit were filtered through a 0.2-µm filter paper (Millipore, Billerica, Mass., USA), and the filter paper was suspended in 10 mL of phosphate-buffered saline (PBS, pH 7.4). Next, 50 µL of the suspension was plated in duplicate on R2A agar (Becton, Dickinson and Company, Sparks, USA) using a spiral plater (IUL, Barcelona, Spain). The plates were incubated at 25°C for 7 days. The colony morphology on the R2A agar was examined and then 3–5 numerically dominant colonies per water sample were selected for pure culture. Pure culture was performed on R2A agar at 25°C for 7 days, and then each colony was subcultured in R2A broth (Becton, Dickinson and Company) at 25°C for 7 days.

Genomic DNA extraction and PCR analysis for the identification of bacteria. One milliliter of the bacterial solution cultivated in the R2A broth was transferred to a 1.5 mL tube and centrifuged at 13,000 × g for 3 min. The supernatant was discarded and suspended in 200 µL of sterile distilled water. Genomic DNA was extracted from the suspension using the G-spin™ Genomic DNA Extraction Kit (Intron Biotechnology, Inc., Gyeonggi-do, Korea) according to the manufacturer’s instructions. The 16S rRNA gene was amplified using PCR primers (27F; 5'-AGA GTT TGA TCM TGG CTC AG-3', 1492R; 5'-GYY TAC CTT GTT AGC ACT T-3') on the GeneAmp PCR System 9700 (PerkinElmer; Waltham, MA, USA) (de Lillo et al., 2006). The 20 µL reactions were prepared containing 0.1 µM of the forward and reverse primers, Accupower HotStart PCR Premix (Bioneer, Deajeon, Korea), sterile distilled water, and 100 pg of DNA template. The PCR conditions were as follows: an initial activation cycle at 94°C for 2 min and 34 cycles at 94°C for 30 s; 55°C for 30 s; 72°C for 1 min. The amplified PCR products were purified according to the manufacturer’s instructions with the AccuPrep® PCR purification kit (Bioneer). The purified PCR products were sent to Cosmogenetech (Seoul, Korea) for nucleotide sequencing of the 16S rRNA gene. All sequences were compared with reference sequences in the genome database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Selection and preparation of bacteria. A total of 56 isolates, including 12 genera, were identified by sequencing, and one strain of each genus was selected for susceptibility testing. The 12 selected isolates were subcultured in R2A broth and cultured at 25°C for 7 days. The cultures were stored at −70°C with 50% glycerol and used for each experiment.

Chemical agents. Four chemical agents that have been used as DUWL disinfectant products were selected for susceptibility testing. These chemical agents are as follows: sodium hypochlorite (Junsei Chemical Co., Ltd, Tokyo, Japan), hydrogen peroxide (Merck, Damstadt, Germany), chlorhexidine gluconate (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), and ethanol (Honeywell, Ulsan, Korea). Additionally, cetylpyridinium chloride (Sigma-Aldrich, St. Louis, Mo., USA) was used in susceptibility testing as a new disinfecting chemical agent for DUWL.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination. The MICs were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines by two-fold serial broth microdilution (Clinical and Laboratory Standards Institute, 2009). In brief, each antimicrobial agent was diluted two-fold serially in a 96-well plate (SPL,
Served and measured. For the determination of MBC, 50-μL agent for inhibiting bacterial growth was visually observed for 7 days under aerobic conditions. The MIC of the chemical agent was determined as the minimal concentration required to kill 99.9% of the bacteria after exposure to the chemical agent. That is, the concentration of chemical agent that failed to regrow bacteria after exposure to the chemical agent. The MIC, MBC, MBIC and MBEC of the 12 isolates differed according to the chemical agent. Differences were considered significant at \( p < 0.05 \). All statistical analyses were performed using a previously reported method (Reiter et al., 2013). In brief, 20 μL of bacterial suspensions at a density of \( 1 \times 10^8 \) CFU/ml were added to 180 μL R2A broth, placed into a 96-well polystyrene flat-bottom microtiter plate (SPL), and incubated at 25°C for 7 days without shaking to allow for bacterial attachment. The broth (containing non-adhered cells) was removed from each well and the plates were rinsed two times with 150 μL PBS. Serial two-fold dilutions of each chemical agent were prepared in a second microtiter plate with R2A broth and were added to each well of the first plate in which the biofilm was formed. The plate was incubated at 25°C for 7 days. After incubation, the lowest chemical agent concentration that showed no growth after exposure to biofilm was recorded as MBIC.

After MBIC measurement, the broth was removed and the wells were washed two times with PBS and then chemical agent-free R2A broth added, followed by incubation at 25°C for 7 days. MBEC was determined as the minimal concentration of chemical agent that failed to regrow bacteria after exposure to the chemical agent. That is, the MBEC is the minimum concentration required to eradicate the biofilm. If the same results were obtained in two repeated experiments, the values were set as the MBIC and MBEC. If the results were different from two experiments, the tests were repeated to determine MBIC and MBEC.

Statistical analysis. The Kruskal-Wallis test and Mann-Whitney test were used to statistically confirm whether the MIC, MBC, MBIC and MBEC of the 12 isolates differed according to the chemical agent. Differences were considered significant at \( p < 0.05 \). All statistical analyses were performed using the Social Sciences (SPSS) software version 23.0 (SPSS, Chicago, IL, USA).

Results

Isolates

A total of 56 strains were isolated from the water samples collected from the dental units (Table 1). The 56 isolates belonged to 3 phyla, 12 genera and 31 species. Of the isolates, 54 isolates belonged to Proteobacteria, which was the dominant phylum. In Proteobacteria, Novosphingobium and Sphingomonas were identified as abundant genera, accounting for 25.9% (14 isolates of the

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Table 1. List of all the species isolated in the 15 dental chair units.

| Phylum           | Genus                | Species                      | Total |
|------------------|----------------------|------------------------------|-------|
| Proteobacteria   | Acidovorax           | Acidovorax delafeldii*       | 1     |
|                  | Capriavidus          | Capriavidus pauculus*        | 1     |
|                  | Methylbacterium      | Methylbacterium aquaticum*   | 1     |
|                  | Novosphingobium      | Novosphingobium aromaticivorans* | 1      |
|                  | Pelomonas            | Pelomonas paraquae*          | 2     |
|                  | Polaromonas          | Polaromonas aquatica*        | 5     |
|                  | Sphingobium          | Sphingobium limneticum*      | 1     |
|                  | Sphingomonas         | Sphingomonas echinoides*     | 7     |
|                  | Sphingopyxis         | Sphingopyxis panaciterrae*   | 2     |
| Bacteroidetes    | Sediminibacterium    | Sediminibacterium salmoneum* | 1     |
| Actinobacteria   | Microbacterium        | Microbacterium testaceum*    | 1     |
| Total            |                      |                              | 56    |

*The selected isolates used for susceptibility tests.

Seoul, South Korea) with R2A broth. It was confirmed that each chemical agent was dissolved in the R2A broth with no precipitate formed in dilutions. The bacterial culture was diluted in R2A broth to a concentration of 0.5 McFarland standard (approximately \( 1 \times 10^8 \) CFU/ml) and was inoculated into each well at final concentrations of 5 \( \times 10^8 \) CFU/ml. The 96-well plates were incubated at 25°C for 7 days under aerobic conditions. The MIC of the chemical agent for inhibiting bacterial growth was visually observed and measured. For the determination of MBC, 50-μL aliquots from wells where there were no visible growth in the MIC experiment were plated onto R2A agar and incubated at 25°C for 7 days. The MBC was measured as the lowest concentration of the chemical agents that killed 99.9% of the bacteria. If the same results were obtained in two repeated experiments, the values were set as the MIC and MBC. If the results were different from two experiments, the tests were repeated to determine MIC and MBC.

Minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) determination. MBIC and MBEC experiments were performed using a previously reported method (Reiter et al., 2013). In brief, 20 μL of bacterial suspensions at a density of \( 1 \times 10^8 \) CFU/ml were added to 180 μL R2A broth, placed into a 96-well polystyrene flat-bottom microtiter plate (SPL), and incubated at 25°C for 7 days without shaking to allow for bacterial attachment. The broth (containing non-adhered cells) was removed from each well and the plates were rinsed two times with 150 μL PBS. Serial two-fold dilutions of each chemical agent were prepared in a second microtiter plate with R2A broth and were added to each well of the first plate in which the biofilm was formed. The plate was incubated at 25°C for 7 days. After incubation, the lowest chemical agent concentration that showed no growth after exposure to biofilm was recorded as MBIC.

After MBIC measurement, the broth was removed and the wells were washed two times with PBS and then chemical agent-free R2A broth added, followed by incubation at 25°C for 7 days. MBEC was determined as the minimal concentration of chemical agent that failed to regrow bacteria after exposure to the chemical agent. That is, the MBEC is the minimum concentration required to eradicate the biofilm. If the same results were obtained in two repeated experiments, the values were set as the MBIC and MBEC. If the results were different from two experiments, the tests were repeated to determine MBIC and MBEC.

Table 2. 16S rDNA gene identification of isolates used for susceptibility testing using GenBank.

| Isolates | GenBank     | Species match | Homology |
|----------|-------------|---------------|----------|
| HY1      | MG763899    | Acidovorax delafeldii | 100%    |
| HY10     | MG763900    | Brevundimonas subvibrioides | 99%    |
| HY12     | MG763901    | Cupriavidus pauculus | 99%    |
| HY14     | MG763902    | Methylbacterium aquaticum | 100%    |
| HY21     | MG763903    | Microbacterium testaceum | 100%    |
| HY25     | MG763904    | Novosphingobium fuchskuhlense | 100%    |
| HY36     | MG763905    | Pelomonas parquae | 99%    |
| HY40     | MG763906    | Polomonas aurata | 100%    |
| HY47     | MG763907    | Sediminibacterium salmoneum | 99%    |
| HY49     | MG763908    | Sphingobium xenophagum | 100%    |
| HY54     | MG763909    | Sphingomonas echinoides | 99%    |
| HY70     | MG763910    | Sphingobium panaciterrae | 100%    |
Table 3. Minimum inhibitory concentration values of the 12 isolates from dental unit waterlines against 5 chemical agents.

| Isolates                    | Sodium hypochlorite (%) | Hydrogen peroxide (%) | Chlorhexidine (µg/mL) | Cetylpyridinium chloride (µg/mL) | Ethanol (%) |
|-----------------------------|-------------------------|-----------------------|-----------------------|----------------------------------|-------------|
| Acidovorax delafieldi       | ≥1                      | 0.0156                | 1.95                  | 1.95                             | 35          |
| Brevundimonas subvibrioides | 0.0156                  | 0.0156                | 0.97                  | 0.12                             | 35          |
| Cupriavidus pauculus        | ≥1                      | 0.0156                | 3.9                   | 15.62                            | 35          |
| Methylbacterium aquaticum   | 0.5                     | 0.0156                | 1.95                  | 0.24                             | 35          |
| Microbacterium testaceum    | 0.5                     | 0.0156                | 3.9                   | 3.9                              | 36          |
| Novosphingobium fuchshulense| 0.125                   | 0.0156                | 1.95                  | 1.95                             | 35          |
| Pelomonas puraquae          | 0.125                   | 0.0156                | 3.9                   | 3.9                              | 8.75        |
| Pseudomonas aquatic         | 0.5                     | 0.0156                | 1.95                  | 0.97                             | 35          |
| Sediminibacterium salmonae | 0.5                     | 0.0156                | 3.9                   | 3.9                              | 35          |
| Sphingobium xenophagum      | ≥1                      | 0.0078                | 1.95                  | 0.97                             | 35          |
| Sphingomonas echinoides     | 0.125                   | 0.0078                | 0.48                  | 0.97                             | 35          |
| Sphingopyxis panaciterrae   | 0.125                   | 0.0156                | 0.97                  | 0.24                             | 17.5        |

54 isolates).

One strain was selected from each genus and a total of 12 isolates were used for the susceptibility tests (Table 2). The isolates used in the susceptibility tests were as follows: Sphingomonas echinoides, Polaromonas aquatic, Novosphingobium fuchshulense, Sphingobium xenophagum, Pelomonas puraquae, Sphingopyxis panaciterrae, Acidovorax delafieldii, Brevundimonas subvibrioides, Cupriavidus pauculus, Methylbacterium aquaticum, Microbacterium testaceum, and Sediminibacterium salmonae.

Susceptibility to sodium hypochlorite

The susceptibilities of the 12 isolates according to each chemical agent are shown in Tables 3–6. The MICs and MBCs of the isolates for sodium hypochlorite were 0.125–0.5% except for B. subvibrioides. For the MIC, N. fuchshulense and Sb. xenophagum were lowest at 0.0625%. For B. subvibrioides, the MBCs of all isolates were greater than 1%. For B. subvibrioides, the MIC and MBC were 16 times higher than the MIC and MBC. In addition, the MIC of most isolates were 1–2 times higher than that of the MIC.

Susceptibility to hydrogen peroxide

In the susceptibility test for hydrogen peroxide, the MICs and MBCs of all isolates were similar in the range of 0.0078–0.0156%. The MBIC and MBEC ranged from 0.0156% to >1%. The MBECs of 7 isolates were greater than 1%. The MBEC (>2%) of Sm. echinoides was 128 times higher than the MIC (0.0078%). The MBECs of 7 isolates (including Sm. echinoides) among the 12 isolates were 64 times higher than the MBC.

Susceptibility to chlorhexidine gluconate

The MIC and MBC of all isolates were 0.97–3.9 µg/ml except for the MIC of Sm. echinoides (0.48 µg/ml) and the MBC of C. pauculus (7.81 µg/ml). For the MIC, except for B. subvibrioides, it ranged from 7.81–15.62 µg/ml. The MBC of Mi. testaceum was above 2,000 µg/ml. In addition, the MBC of Mi. testaceum was 512 times higher than the MBC (3.9 µg/ml).

Susceptibility to cetylpyridinium chloride

The lowest MIC and MBC for cetylpyridinium chloride were found in B. subvibrioides at 0.12 µg/ml and 0.24 µg/ml, respectively, and this MIC and MBC were the lowest among the MIC and MBC of 12 isolates for 5 chemicals. The MIC of cetylpyridinium chloride for C. pauculus was the highest at 15.62 µg/ml. The MBC, MBIC, and MBEC of C. pauculus were also higher than those of other isolates (15.62 µg/ml, 31.25 µg/ml, and 250 µg/ml, respectively). The MBIC of Me. aquaticum and the MBC of Se. salmonae were lowest at 0.97 µg/ml and 15.62 µg/ml, respectively, and these two concentrations were also the lowest among the MBIC and MBEC of 5 chemicals.
and 12 isolates. The MBIC of Sp. panaciterrae and B. subvibrioides (31.25 μg/mL and 15.62 μg/mL, respectively) were 128 times higher than the MIC, and the MBEC (both 62.5 μg/mL) was 256 times higher than the MBC. The MIC, MBC, MBIC and MBEC of 12 isolates for cetylpyridinium chloride were not statistically different from those of all isolates were the same as the MIC, and the MBIC of Me. aquaticum were less than 35%. For MBEC, all isolates except for Pe. puraquae were lowest (both 8.75%).

### Susceptibility to ethanol

In ethanol, the MIC, MBC, and MBIC of all isolates were less than 35%. For MBEC, all isolates except for Me. aquaticum and Pe. puraquae were over 70%. The MIC and MBC of Pe. puraquae were lowest (both 8.75%). Except for Pe. puraquae and Sp. panaciterrae, the MBICs of all isolates were the same as the MIC, and the MBIC of Pe. puraquae and Sp. panaciterrae were 4 and 2 times higher than the MIC, respectively.

### Discussion

The biofilms formed on the inner surface of the DUWL provides a continuous reservoir for bacteria (Williams et al., 1995). Therefore, DUWL disinfectant products should be able to remove or reduce the DUWL biofilms. As a standard method to confirm the susceptibility of bacteria to antimicrobial agents, MIC and MBC are performed on planktonic bacteria (Clinical and Laboratory Standards Institute, 2009). However, the application of the MIC for the removal of biofilms was ineffective, and the MBEC was proposed to evaluate the susceptibility of the bacteria to the antimicrobial agents in the biofilms state (Anwar et al., 1990; Ceri et al., 1999). So far, there have been many studies comparing MICs with MBECs, and previous studies have demonstrated that MBECs are 10- to 1000-fold greater than MICs (Ceri et al., 1999; Reiter et al., 2013; Sandoe et al., 2006).

R2A was used for the cultivation of isolates and for the identification of susceptibility tests in our experiments. Although the DUWL isolates were detected in water, R2A was used for the experiments because the growth rates of the isolates were very slow when cultured in water. The low-nutrient medium, R2A, is considered to be a suitable medium for culturing bacteria grown in a low-nutrient environment such as DUWL (Bartoloni et al., 2006; Porteous et al., 2013).

One strain per genus for a total of 12 isolates used in the susceptibility tests were selected from 56 isolates that were originally isolated. Although many studies have identified bacterial diversity in DUWLs, most studies have identified bacteria in DUWLs using cultivation techniques, and the results differ for each study (Barbeau et al., 1996; Yabune et al., 2008). Costa et al. (2015) confirmed the diversity of DUWL bacteria using pyrosequencing analysis. The profile of the total bacteria in the DUWL was confirmed in Costa's study, but the species level of the detected bacteria was unknown. For this reason, repre-

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### Table 5. Minimum biofilm inhibitory concentrations of the 12 isolates from dental unit waterlines against 5 chemical agents.

| Isolates               | Sodium hypochlorite (μg/mL) | Hydrogen peroxide (μg/mL) | Chlorhexidine (μg/mL) | Cetylpyridinium chloride (μg/mL) | Ethanol (%) |
|------------------------|-----------------------------|---------------------------|-----------------------|----------------------------------|------------|
| Acidovorax delafielde | ≥1                          | 0.0156                    | 7.81                  | 3.9                              | 35         |
| Brevundimonas subvibrioides | 0.25                      | 0.0312                    | 62.5                  | 15.62                            | 35         |
| Cupriaviridia pauculus | ≥1                          | 0.5                       | 15.62                 | 31.25                            | 35         |
| Methylbacterium aquaticum | 0.5                       | 0.25                       | 7.81                  | 0.97                             | 35         |
| Microbacterium testaceum | 0.5                       | 0.5                       | 7.81                  | 15.62                            | 35         |
| Novosphingobium fuchskuhlense | 0.0625                   | 0.0156                    | 7.81                  | 7.81                             | 35         |
| Pelomonas puraquae     | 0.125                      | 0.0156                    | 7.81                  | 15.62                            | 35         |
| Polaromonas aquaticia  | 0.5                        | 0.0312                    | 7.81                  | 7.81                             | 35         |
| Sediminibacterium salmonene | 0.5                     | 0.0156                    | 7.81                  | 1.9                              | 35         |
| Sphingobium xenophagum | 0.0625                     | 0.25                       | 15.62                 | 15.62                            | 35         |
| Sphingomonas echinoides | 0.25                       | 0.0625                    | 15.62                 | 15.62                            | 35         |
| Sphingopyxis panaciterrae | ≥1                       | 0.0156                    | 7.81                  | 31.25                            | 35         |

### Table 6. Minimum biofilm eradication concentrations of the 12 isolates from dental unit waterlines against 5 chemical agents.

| Isolates               | Sodium hypochlorite (μg/mL) | Hydrogen peroxide (μg/mL) | Chlorhexidine (μg/mL) | Cetylpyridinium chloride (μg/mL) | Ethanol (%) |
|------------------------|-----------------------------|---------------------------|-----------------------|----------------------------------|------------|
| Acidovorax delafielde | ≥1                          | ≥4                        | 62.5                  | 62.5                             | ≥70        |
| Brevundimonas subvibrioides | 0.25                    | 0.0312                    | 62.5                  | 62.5                             | ≥70        |
| Cupriaviridia pauculus | ≥1                          | ≥4                        | 125                   | 250                             | ≥70        |
| Methylbacterium aquaticum | ≥1                        | 0.5                       | 62.5                  | 31.25                            | 35         |
| Microbacterium testaceum | ≥1                        | ≥4                        | ≥4000                 | 125                             | ≥70        |
| Novosphingobium fuchskuhlense | ≥1                     | 0.0312                    | 31.25                 | 15.62                            | ≥70        |
| Pelomonas puraquae     | ≥1                          | 0.125                     | 15.62                 | 31.25                            | ≥70        |
| Polaromonas aquaticia  | ≥1                          | ≥4                        | 250                   | 62.5                             | ≥70        |
| Sediminibacterium salmonene | ≥1                     | 0.0312                    | 7.81                  | 15.62                            | ≥70        |
| Sphingobium xenophagum | ≥1                          | 1                         | 125                   | 31.25                            | ≥70        |
| Sphingomonas echinoides | ≥1                          | ≥4                        | 31.25                 | 31.25                            | ≥70        |
| Sphingopyxis panaciterrae | ≥1                       | ≥4                        | 62.5                  | 62.5                             | ≥70        |
sentative bacterial species among the bacteria found in DUWL cannot be determined yet. Similar to the results of Costa et al., the dominant phylum of the isolates in our study was Proteobacteria. And among the 12 isolates used in our susceptibility test, the genera of 11 isolates were included in the list of bacteria in the DUWL identified through previous pyrosequencing studies (Costa et al., 2015).

In the biofilm state, the susceptibilities of the isolates to chemical agents were different from their respective planktonic state. For example, most isolates showed high susceptibility to chlorhexidine, but *M. testaceum* showed a MBEC over 4,000 µg/ml and was particularly less susceptible than other isolates. In the case of sodium hypochlorite, *B. subvibrioides* was more susceptible than other isolates. The different susceptibilities of the isolates to the same chemical agent may be due to the different characteristics of biofilm development depending on each isolate (Schwering et al., 2013). One study reported that bacterial susceptibility to antimicrobial agents may be influenced by their biofilm formation ability, and that the greater the ability, the greater the resistance to antimicrobial agents (Antunes et al., 2011). The ability of bacteria to adhere to the surface is also considered to be the greatest factor affecting the resistance to antimicrobial agents (LeChevallier et al., 1988). Therefore, further studies are needed to confirm the bacterial ability to adhere and to form biofilms on the DUWL.

Many disinfectant products are being used to disinfect DUWL, and the efficacies of disinfectant products have been confirmed through previous studies (Karpay et al., 1999; Kettering et al., 2002; Lin et al., 2011; Schel et al., 2006; Walker et al., 2003). Sodium hypochlorite (0.5%) and hydrogen peroxide (1%) are commonly used chemical agents for DUWL disinfectant products (Karpay et al., 1999; Lin et al., 2011). A mixture of chlorhexidine and ethanol is used as a DUWL disinfectant product, containing 0.12% (1,200 µg/ml) and 12%, respectively (Kettering et al., 2002; Schel et al., 2006; Walker et al., 2003). A few studies have reported that disinfectant products containing sodium hypochlorite or hydrogen peroxide are better than disinfectant products containing chlorhexidine (Kettering et al., 2002; Schel et al., 2006). However, in our experiment, the MBECs of 11 isolates were greater than 0.5% for sodium hypochlorite, and for hydrogen peroxide the MBECs of 6 isolates were greater than 2%. In the case of ethanol, the MBECs of DUWL isolates were 35% or 70%, and the susceptibilities of isolates to ethanol were low. On the other hand, the MBECs of most of the DUWL isolates against chlorhexidine were lower than 1,200 µg/ml, and the MBECs of the DUWL isolates were lowest in chlorhexidine compared with sodium chloride, hydrogen peroxide, and ethanol. Therefore, the DUWL isolates were most susceptible to chlorhexidine. Although our isolates were found in the DUWL biofilms of previous studies (Barbeau et al., 1996; Costa et al., 2015; Yabune et al., 2008), the results may be different between previous studies and our study because the bacterial species constituting the DUWL biofilms used in previous studies may be different from the bacterial species used in our experiments, and because the characteristics of each isolate may be different in multi-species and single-species biofilms.

Until now, DUWL disinfectant products containing cetylpyridinium chloride have not been used. The susceptibility test for cetylpyridinium chloride was carried out to confirm the possibility of a new chemical agent for disinfecting DUWLs. Our results showed that the susceptibilities of planktonic and biofilm bacteria to cetylpyridinium chloride were similar to that observed with chlorhexidine. Cetylpyridinium chloride also showed the highest susceptibility among the five chemical agents used in the test.

The susceptibility of isolates to the chemical agents identified in our experimental results can be applied for the development of new DUWL disinfectant products. Cetylpyridinium chloride, which shows the highest susceptibility in our experiments, can be used for disinfecting DUWL and can be utilized in the development of new disinfectant products with better efficacy with chlorhexidine, which has a similar susceptibility. In the future, based on these experimental results, it will be necessary to find a variety of mixtures of chemical agents that increase the effectiveness of removing and reducing DUWL biofilms.

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