An Assessment of the Bacterial Diversity found in Dental Unit Waterlines using the Illumina MiSeq

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Water from the waterlines of dental units is often contaminated with bacteria but there have been few studies accurately assessing the diversity of these bacterial populations. The aim of our study was to assess the bacterial diversity present in water collected from dental unit waterlines using the Illumina MiSeq. Water was collected from two separate dental units located in a dental hospital and two units found in two separate private clinics in Gangneung-si, Korea. From the four water samples that were analyzed, a total of 233 bacterial genera were identified. The most abundant genera were Sphingomonas (25%), Halomonas (20%), Reyranella (8%), and Novosphingobium (6%). Halomonas was more prevalent in the two dental units located at the dental hospital, while Reyranella and Sphingomonas were more commonly found in the private dental clinics. Only 19 of the 233 identified genera were common between water samples from all dental units. Opportunistic pathogens were shown to account for 7.7% of the total bacterial genera identified. Our results have demonstrated that there is a wide assortment of bacterial genera present in dental unit waterlines.

Key words : Infection control / Pathogens / High-Throughput DNA Sequencing / Sphingomonas / Water microbiology.

INTRODUCTION

Guidelines outlined by the Centers for Disease Control and Prevention (CDC) recommend that bacterial counts in water from dental unit waterlines (DUWLs) should not exceed 500 colony-forming units (CFUs)/mL (Kohn et al., 2004). More stringent standards have been established by the American Dental Association (ADA), specifying no more than 200 CFU/mL for DUWLs (1999). Despite these guidelines, water discharged from DUWLs can still be heavily contaminated with microorganisms (Barbeau et al., 1996; Walker et al., 2004; Williams et al., 1993; Yoon and Lee, 2015). The range of microorganisms that have been identified includes heterotrophic water bacteria, opportunistic pathogens, and bacteria of human origin (Al-Hiyasat et al., 2007; Dutil et al., 2007; Walker et al., 2000). As DUWL water is used in most dental treatments, it may be a potential source of infection for both staff and patients. Many studies have examined the bacterial contamination present in DUWLs but few have examined the types of microorganisms in detail (Barbeau et al., 1996; Costa et al., 2015; Szymanska and Sitkowska, 2013a; Walker et al., 2004; Yabune et al., 2008). In addition, most of these studies have assessed the bacterial diversity of water from DUWLs using in vitro cultivation (Barbeau et al., 1996; Szymanska and Sitkowska, 2013a; Yabune et al., 2008). These cultivation techniques are limited in that they cannot include bacteria that are unable to adapt to culture. Therefore, data examining bacterial diversity in water from DUWLs is incomplete. An alternate, culture-independent method that utilizes molecular techniques and 16S rRNA sequences to identify bacteria overcomes the drawbacks found in previous studies (Jeon et al., 2007; Singh et al., 2003). By avoiding culturing bottlenecks, these molecular techniques provide a more complete picture of the diversity present in the microbial community of DUWLs. High-throughput DNA sequencing method have been successfully used...
in several microbiological studies (Chun et al., 2010; Holinger et al., 2014; Poza et al., 2012). To date, there has only been one prior study that has used high-throughput DNA sequencing to examine bacterial diversity in water from DUWLs (Costa et al., 2015). Comparisons between this study and culture-dependent methods have shown inconsistent results when assessing bacterial diversity in water from DUWLs. This indicates that additional studies are needed to accurately distinguish core bacterial species. In our present study, we have investigated the bacterial diversity in water discharged from DUWLs using Illumina MiSeq, one of the high-throughput DNA sequencing methods, contributing to a better understanding of bacterial diversity in this potential source of infection.

**MATERIALS AND METHODS**

**Sampling**
Two dental units (A, B) from a dental hospital and two further dental units (C, D) from two different private dental clinics in Gangneung-si, Korea were used in this study. Before sampling, the inlet and handle of the waterline were cleaned with 70% ethanol to minimize contamination by external bacteria. A 500 mL sample of water from each ultrasonic scaler was collected using sterilized 1 L glass bottles. To neutralize any residual chlorine, 10% sodium thiosulphate solution (Yakuri Pure Chemicals Co., Ltd., Kyoto, Japan) was added to each sample. Samples were then transported directly to the laboratory for analysis.

**Total number of heterotrophic bacteria**

To assess the total number of heterotrophic bacteria in the water samples, 50 µL from each was plated onto R2A agar (Becton, Dickinson and Company, Sparks, USA) in duplicate using an automatic spiral plater (IUL, S.A., Barcelona, Spain). Plates were incubated for seven days at 28 °C, and the colony forming units per mL (CFU/mL) were calculated.

**DNA extraction**

Samples were filtered through a 0.2 µm pore-size filter (Millipore, Billerica, MA, USA) to isolate bacteria. The filters were then placed into sterilized conical tubes containing 0.15 mm glass beads and 10 mL of phosphate buffered saline (PBS) and vortexed (Walker et al., 2000). Genomic DNA in the PBS suspension was then extracted using a G-spin Genomic DNA Extraction Kit (Intron Biotechnology Inc., Seongnam, Korea) following the manufacturer’s instructions.

**PCR amplification and Illumina sequencing**

PCR amplification was performed on extracted DNA using primers targeting the V3 and V4 regions of the 16S RNA gene. The primers used were 341F (5'-TCGTCCGACGCTATATATAGTGCAAGACAGCCTACGGGNGGCWGCAG-3'; underlining sequence indicates the target region primer) and 805R (5'-GTCTCGTGGGCTCGG-AGATGTGTATAAGACAGAGACTACHVGGGTATCTAATCC-3'). PCR amplification was performed using an initial denaturation step at 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 sec, primer annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec, with a final elongation step at 72 °C for 5 min. Secondary amplification to attach the Illumina NexTera barcode was performed using a i5 forward (5'-AATGATACGGCGACACGATCTACAC-XXXXXXX-TCGTCCGACGCCGTC-3'; X indicates the barcode region) and i7 reverse primer (5'-CAA GCAGAGACACGGATCATCGAGAT-XXXXXXX-AGTC TCATGGGCTCTCGG-3'). The conditions of the secondary amplification were the same as the first PCR, but with only eight amplification cycles. PCR product was confirmed using 2% agarose gel electrophoresis and visualized with a Gel Doc system (BioRad, Hercules, CA, USA). Amplified products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Equal concentrations of purified products were then pooled together and short fragments removed (non-target products) using Ampure beads kit (Agencourt Bioscience, MA, USA). The quality and sizes of products were assessed using a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using a DNA 7500 chip (Agilent). Mixed amplicons were pooled and sequencing was carried out at Chunlab, Inc. (Seoul, Korea) using an Illumina MiSeq Sequencing system (Illumina, USA), according to the manufacturer’s instructions.

**Sequencing data analysis**

Analysis was conducted following a methodology outlined in other studies (Chun et al., 2010; Hur et al., 2011; Kim et al., 2012a). Briefly, reads obtained from different samples were sorted by the barcodes unique to each PCR product. Barcode, linker, and primer sequences were removed from the sequencing reads. Any reads containing two or more ambiguous nucleotides, low quality score (average score < 25), or were shorter than 300 bp, were discarded. Sequences are denoised using DUDE-Seq (Eddy, 2011) and Potential chimeric sequences were detected using Bellerophon and removed (Huber et al., 2004). A taxonomic classification for each read was then assigned from the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net).
containing 16S rRNA sequences from strains that have valid published names. We used the following sequence similarity cut-offs for the taxonomic assignment of each read (x = sequence similarity to reference sequences): species (x \geq 97\%), genus (97> x \geq 94.5\%), family (94.5> x \geq 86.5\%), order (86.5> x \geq 82\%), class (82> x \geq 78.5\%), and phylum (78.5> x \geq 75\%).

Assessment of pathogenic bacteria
An assessment of the proportion of pathogenic bacteria present in each sample was performed using a previously described method (Costa et al., 2015). The bacterial genera identified by Illumina MiSeq sequencing analysis were then investigated using literature searches on the PubMed database. To identify relevant published case studies examining pathogenic bacteria at the species level, we only used bacterial species known to be detected in water from DUWLs. In addition, we limited searches to case studies published in the last decade.

RESULTS

Number of total heterotrophic bacteria
The number of bacteria in all of the DUWL water samples was found to greatly exceed 200 CFU/mL, with a mean of 1.9x10^5 CFU/mL (values ranged from 1.8x10^4 CFU/mL to 6.6x10^5 CFU/mL) across the four dental units.

Bacterial diversity in dental units
Sequencing of the dental unit water samples resulted in 19,774, 19,331, 19,735, and 18,678 valid reads for unit A, B, C, and D, respectively. A total of 233 bacterial genera were identified across the water samples from the four dental units (Table 1). When examining the total diversity across the four samples, the most abundant genera were Sphingomonas (25\%), Halomonas (20\%), Reyranella (8\%), and Novosphingobium (6\%) (Fig. 1).

Proteobacteria was the most dominant phylum in four dental units (87.3\%, 83.2\%, 84.5\% and 88.5\% for unit A, B, C and D, respectively). At the genus level,
### TABLE 1. List of all the genera identified in the dental unit waterlines using the Illumina MiSeq.

| Phylum Class Genus       | Unit A | Unit B | Unit C | Unit D |
|--------------------------|--------|--------|--------|--------|
| Acidobacteria Acidobacteria Koribacter | 0      | 0      | 3      | 0      |
| Blastocatellia Acidobacteria Aridibacter | 0      | 3      | 20     | 0      |
| Blastocatella Acidobacteria Stenotrophobacter | 2      | 9      | 4      | 0      |
| Holophagae Holophaga | 28     | 24     | 0      | 0      |
| Solibacteres Bryobacter | 0      | 0      | 95     | 0      |
| Paludibaculum Solibacter | 3      | 6      | 0      | 7      |
| Paludibaculum Solibacter | 262    | 227    | 0      | 0      |
| Actinobacteria Actinomycetes Aquihabitans | 1      | 0      | 0      | 0      |
| Actinobacteria Actinomyces | 3      | 0      | 0      | 0      |
| Corynebacterium Dietzia | 2      | 1      | 1      | 0      |
| Mycobacterium Nocardia | 2      | 0      | 0      | 34     |
| Geoematophilus Herbiconius | 0      | 0      | 1      | 0      |
| Limnoluna Lysinimonas | 0      | 0      | 414    | 27     |
| Marisediminicola Microbacterium | 1      | 0      | 0      | 0      |
| Micrococcus Planktophila | 123    | 84     | 11     | 0      |
| Propionibacterium Propionibacterium | 0      | 5      | 0      | 0      |
| Rubrobacteria Gaiella | 1      | 3      | 2      | 0      |
| Armatimonadetes Chthonomonadetes; Chthonomonas | 0      | 2      | 0      | 0      |
| Fimbriomonadia Fimbritimonas | 0      | 12     | 0      | 0      |
| Bacteroidetes Bacteroidia Bacteroides | 0      | 9      | 0      | 1      |
| Porphyromonas Alloprevotella | 0      | 3      | 1      | 0      |
| Prevotella Cytophaga | 1      | 0      | 1      | 0      |
| Alloprevotella Cytophaga | 0      | 13     | 0      | 0      |
| Predobacter Cytophaga | 2      | 0      | 0      | 0      |
| Spirosoma Flavobacteria | 10     | 0      | 0      | 0      |
| Capecocytobacteria Sphingobacteria | 0      | 0      | 1      | 0      |
| Ferruginibacter Helminias | 29     | 8      | 6      | 0      |
| Hydrotalea Hydrotalea | 90     | 11     | 0      | 0      |
| Lacibacter Parafilimonas | 43     | 33     | 30     | 0      |
| Parafilimonas Sediminibacterium | 0      | 6      | 0      | 0      |
| Sediminibacterium Aquirestis | 212    | 15     | 22     | 1525   |
| Chlamydiae Chlamydiae | Metachlamydia | 1      | 0      | 46     | 1      |
| Acidimicrobia Actinobacteria Aquihabitans | 1      | 0      | 0      | 0      |
| Actinobacteria Actinomyces | 3      | 0      | 0      | 0      |
| Corynebacterium Dietzia | 2      | 1      | 1      | 0      |
| Mycobacterium Nocardia | 2      | 0      | 0      | 34     |
| Geoematophilus Herbiconius | 0      | 0      | 1      | 0      |
| Limnoluna Lysinimonas | 0      | 0      | 414    | 27     |
| Marisediminicola Microbacterium | 1      | 0      | 0      | 0      |
| Micrococcus Planktophila | 123    | 84     | 11     | 0      |
| Propionibacterium Propionibacterium | 0      | 5      | 0      | 0      |
| Rubrobacteria Gaiella | 1      | 3      | 2      | 0      |
| Armatimonadetes Chthonomonadetes; Chthonomonas | 0      | 2      | 0      | 0      |
| Fimbriomonadia Fimbritimonas | 0      | 12     | 0      | 0      |
| Bacteroidetes Bacteroidia Bacteroides | 0      | 9      | 0      | 1      |
| Porphyromonas Alloprevotella | 0      | 3      | 1      | 0      |
| Prevotella Cytophaga | 1      | 0      | 1      | 0      |
| Alloprevotella Cytophaga | 0      | 13     | 0      | 0      |
| Predobacter Cytophaga | 2      | 0      | 0      | 0      |
| Spirosoma Flavobacteria | 10     | 0      | 0      | 0      |
| Capecocytobacteria Sphingobacteria | 0      | 0      | 1      | 0      |
| Ferruginibacter Helminias | 29     | 8      | 6      | 0      |
| Hydrotalea Hydrotalea | 90     | 11     | 0      | 0      |
| Lacibacter Parafilimonas | 43     | 33     | 30     | 0      |
| Parafilimonas Sediminibacterium | 0      | 6      | 0      | 0      |
| Sediminibacterium Aquirestis | 212    | 15     | 22     | 1525   |
| Chlamydiae Chlamydiae | Metachlamydia | 1      | 0      | 46     | 1      |
| Phylum     | Class     | Genus       | Unit A | Unit B | Unit C | Unit D |
|------------|-----------|-------------|--------|--------|--------|--------|
| Phylum     | Class     | Genus       | Unit A | Unit B | Unit C | Unit D |
| Phylum     | Class     | Genus       | Unit A | Unit B | Unit C | Unit D |

**BACTERIAL DIVERSITY IN DENTAL UNIT WATERLINES**

- **Phylum:** Paranaedibacter
  - **Class:** 0 5 0 119
- **Phylum:** Rickettsia
  - **Class:** 32 20 0 0
- **Phylum:** Pelagibacter
  - **Class:** 4 0 4 0
- **Phylum:** Altererythrobacter
  - **Class:** 1 0 185 0
- **Phylum:** Erythrobacter
  - **Class:** 0 0 1 0
- **Phylum:** Blastomonas
  - **Class:** 0 0 1 0
- **Phylum:** Novosphingobium
  - **Class:** 1275 218 1094 516
- **Phylum:** Paraphosphoryx
  - **Class:** 0 1 0 0
- **Phylum:** Rhizohabdecus
  - **Class:** 1 96 2 125
- **Phylum:** Sandarakinorhabds
  - **Class:** 19 12 0 1
- **Phylum:** Sphingobium
  - **Class:** 1222 225 841 84
- **Phylum:** Sphingomonas
  - **Class:** 322 1081 1219 1101
- **Phylum:** Sphingopyx
  - **Class:** 0 121 14 1
- **Phylum:** Sphingorhabdus
  - **Class:** 72 187 0 1
- **Phylum:** Sphingosinilacina
  - **Class:** 0 2 0 0

- **Phylum:** Betaproteobacteria
  - **Class:** Alcaligenes
    - **Genus:** Acidovorax
      - **Unit A:** 117 49 1 502
    - **Genus:** Albidiferax
      - **Unit A:** 0 3 0 0
    - **Genus:** Brachymonas
      - **Unit A:** 1 0 0 1
    - **Genus:** Caenimonas
      - **Unit A:** 8 0 0 0
    - **Genus:** Curvibacter
      - **Unit A:** 6 5 0 0
    - **Genus:** Delfia
      - **Unit A:** 0 3 0 0
    - **Genus:** Hydrogenophaga
      - **Unit A:** 0 1 0 0
    - **Genus:** Limnohabitans
      - **Unit A:** 0 43 0 0
    - **Genus:** Ottowia
      - **Unit A:** 0 1 0 0
    - **Genus:** Polaromonas
      - **Unit A:** 0 133 235 0
    - **Genus:** Ramlibacter
      - **Unit A:** 0 2 0 0
    - **Genus:** Variorax
      - **Unit A:** 0 1 0 0
    - **Genus:** Massilia
      - **Unit A:** 11 0 0 0
    - **Genus:** Undibacterium
      - **Unit A:** 0 58 0 0
    - **Genus:** Cupriavidus
      - **Unit A:** 68 0 0 21
    - **Genus:** Polynucleobacter
      - **Unit A:** 0 23 0 0
    - **Genus:** Ralstonia
      - **Unit A:** 1 0 3 0
    - **Genus:** Aquabacterium
      - **Unit A:** 263 4 1 0
    - **Genus:** Aquincola
      - **Unit A:** 0 0 18 0
    - **Genus:** Ideonella
      - **Unit A:** 0 0 12 0
    - **Genus:** Kinneretia
      - **Unit A:** 0 0 2 0
    - **Genus:** Methylbium
      - **Unit A:** 0 2 0 0
    - **Genus:** Mitsugia
      - **Unit A:** 0 3 0 0
    - **Genus:** Pelomonas
      - **Unit A:** 0 0 0 153
    - **Genus:** Rhizobacter
      - **Unit A:** 1 204 0 0
    - **Genus:** Roseateles
      - **Unit A:** 0 0 31 0
    - **Genus:** Rubrivivax
      - **Unit A:** 0 0 1 0
    - **Genus:** Gallionella
      - **Unit A:** 8 2 0 0
    - **Genus:** Nitrofoga
      - **Unit A:** 0 1 0 0
Halomonas (36.8%) and Dechloromonas (9.6%) were the main genera in unit A. Halomonas (42.1%) and Phreatobacter (13.2%) dominated the phylum of Proteobacteria in unit B. Reyranella (34.7%) and Sphingomonas (11.4%) appeared to be the dominant species in unit C. Sphingomonas (65.7%) and Sediminibacterium (8.9%) were the main genera in unit D (Fig. 2).

The numbers of bacterial genera in units A, B, C and D were 122, 119, 116 and 51 genera, respectively (Table 2), and there were 19 genera that were common to water samples from all four dental units (Fig. 3). These were Sphingomonas, Halomonas, Reyranella, Novosphingobium, Sphingobium, Dechloromonas, Sediminibacterium, Bradyrhizobium, Methyllobacterium, Acidovorax, Phenyllobacterium, Hyphomicrobium, Mycobacterium, Rhizorhabdus, Afipia, Defluviimonas, Rhodobacter, Gemmata, and Protochlamydia. These 19 genera accounted for 15.6%, 16.4%, 18.6%, and 37.3% of the total bacteria genera for unit A, B, C, and D, respectively. The number of genera shared between units A and B was 65 genera, while 29 were shared between units C and D.

A rarefaction curve is shown in Fig. 4 to compare species diversity of bacteria in each water sample. Operational taxonomic unit (OTU) refers to the individual or group of organisms to be classified and generally refers to species. The slope of the rarefaction curve represents the diversity index. In our experimental data, it can be seen that there are various microbial communities in units A and B.
Pathogenic bacteria

Among the 233 bacteria genera identified in our study, 85% were gram-negative (Table 3). These included ten opportunistic pathogens that made-up 5.1% of the total number of gram-negative bacteria (Table 3). There were 8 gram-positive opportunistic pathogens, which constituted 22.2% of this group of bacteria (Table 3). The total number of opportunistic pathogens was therefore found to be 18, or 7.7% of the total isolated bacteria genera. Finally, oral bacteria were also found in the four water samples (Table 1, 2), including Actinomyces, Porphyromonas, Prevotella, Lactobacillus, and Streptococcus.

DISCUSSION

Our study has used Illumina MiSeq sequencing to characterize the diversity of bacteria present in water...
from DUWLs. Although the four analyzed dental units were located in the same city, Gangneung, there were significant differences in their bacterial diversities. Dental units A and B were located in a pedodontics and a periodontology department of a dental hospital and dental units C and D were located in two different private dental clinic. The bacterial diversities found in water from dental units A and B were similar, while dental unit C had a distinct bacterial diversity from dental units A (or B) and D.

All four dental units were using tap water. To identify the source of microorganisms from the DUWL, Illumina MiSeq sequencing analysis of tap water was performed. However, the analysis was unsuccessful due to the low bacterial DNA concentration in the sample (data not shown). In these dental units, microorganisms in DUWL might have a high probability of being derived from tap water. The microorganisms present in tap water may have established bacterial contamination of DUWL, while growing and forming biofilms depending on the situation of each dental unit, such as temperature and water stagnation time in DUWL, etc. We think further research is needed to clarify the exact origin of microorganisms in DUWL.

Previous studies performed in Canada using culturing techniques have confirmed that there can be significant contamination in water from DUWLs, and *Sphingomonas paucimobilis*, *Acinetobacter calcoaceticus*, *Methyllobacterium mesophilicum*, and *Pseudomonas aeruginosa* were the predominant bacterial species (Barbeau et al., 1996). Yabune et al. (2008) have also shown that the dominant bacteria in water from DUWLs located in a Japanese dental hospital were also *Sphingomonas paucimobilis*, *Acinetobacter haemolytics*, and *Methyllobacterium mesophilicum*. A more recent study using high-throughput DNA sequencing techniques has more comprehensively characterized the bacterial diversity of water from DUWLs in France (Costa et al., 2015), showing frequent contamination by *Sphingomonas* and *Halomonas*. Bacteria of the *Sphingomonas* genus are commonly found in water from DUWLs, although the proportions differ slightly depending on the study. In our study, we found that *Acinetobacter* spp. made up less than 1% of the bacteria identified, counter to observations by Yabune et al. (2008) and Barbeau et al. (1996) that found a high proportion were this genus. Although bacterial diversity is likely to be different depending on the study, identification of *Sphingomonas* and *Methyllobacterium* are common to each study and likely represent the core bacterial genera found in water from DUWLs. The most abundant genus, *Sphingomonas*, is an aerobic gram-negative rod that can be found in a wide-range of environments, including water distribution systems and even seawater. Some *Sphingomonas* spp. (especially, *Sphingomonas paucimobilis*) are implicated in human disease (Ryan and Adley, 2010). *Halomonas*, the second most commonly identified genus, are gram-negative rod-shaped cells typically found in water sources with high salinity (Kim et al., 2010). Some species of *Halomonas* also have pathogenic potential to infect humans (Stevens et al., 2009).

Streptococci are predominantly found in the oral cavity (Ahn et al., 2011). However, in this study,
TABLE 2. The genus of bacteria identified in each dental unit.

| Unit | A                      | B                      | C                      | D                      |
|------|------------------------|------------------------|------------------------|------------------------|
|      | Accumulibacter         | Acidiferoberoberobacter | Acidovorax             | Acidovorax             |
|      | Acidibacter            | Acidovorax             | Acinetobacter          | Afiia                  |
|      | Acidovorax             | Acinetobacter          | Aeromonas              | Akkermansia            |
|      | Acinetobacter          | Afiia                  | Altererythrobacter     | Alloprevotella         |
|      | Actinomycetes          | Akkermansia            | Anaerosporobacter      | Azospira               |
|      | Afiia                  | Albidiferox            | Anaerostipes           | Bacteroides            |
|      | Altererythrobacter     | Alcaligenes            | Aquabacterium          | Brachymonas            |
|      | Anaerobrobacter        | Algoirphagus           | Aquamincola            | Bradyrhizobium         |
|      | Aquabacterium          | Anoxybacillus          | Aridibacter            | Bryobacter             |
|      | Aquicella              | Aquicella              | Bacillus               | Caulobacter            |
|      | Aquihabitsans          | Aquirestis             | Blastocatella          | Clostridium            |
|      | Azospiillum            | Aquirna                | Blastomonas            | Cupvindavus            |
|      | Bacillus               | Aridibacter            | Blautia                | Dechlorivomomas        |
|      | Badoilovibrio          | Azospiella             | Bosea                  | Defluvimonas           |
|      | Blastocatella          | Bacteroides            | Bradyrhizobium         | Desulfonaturnum        |
|      | Brachymonas            | Blastocatella          | Capnocytophaga         | Escherichia            |
|      | Brachyribizum          | Biautia                | Caulobacter            | Faecalibacter          |
|      | Breundimmonas          | Bosea                  | Cavicella              | Gemmata                |
|      | Caenimmonas            | Brachyribizum          | Clostridium            | Halomonas              |
|      | Campylobacter           | Brevundimmonas         | Defluvimonas           | Helicobacter           |
|      | Chlatalinorovans       | Chthonomonas           | Dietzia                | Helmolina              |
|      | Corynebacterium        | Corynebacterium        | Dyerella               | Magnetospinilum         |
|      | Cupriavidus             | Curvibacter            | Enteroberacter         | Metachlamydia          |
|      | Curvibacter             | Dechoromonas           | Erwinia                | Methylobacterin        |
|      | Dechloromonas           | Defluvimonas           | Erythrobacter          | Mycobacterin           |
|      | Deffovimonas           | Delfia                 | Escherichia            | Novosphingobium        |
|      | Devosia                | Devosia                | Eubacterin             | Ochrombactrum          |
|      | Dongia                 | Dyadobacter            | Faecalibacterin        | Paludbaculum           |
|      | Elstera                | Enteroococcus          | Ferruginibacter        | Paracaelibacter         |
|      | Enhydrobacter           | Enterococcus           | Fimbirimonas           | Pelomonas              |
|      | Exigubacterin          | Enterococcus           | Filmbacterin           | Phenyllobacterin       |
|      | Faecalibacterin        | Escherichia            | Filmbacterin           | Phreobacterin          |
|      | Ferribacterin          | Enterococcus           | Filmbacterin           | Proteobacteris         |
|      | Ferruginibacter         | Enteroococcus          | Filmbacterin           | Pseudomonas            |
|      | Filmbacterin           | Enteroococcus          | Filmbacterin           | Reynella               |
|      | Frigidibacter           | Enteroococcus          | Filmbacterin           | Rhizobium              |
|      | Fritschea              | Enteroococcus          | Filmbacterin           | Rhizorhabdus           |
|      | Gaiaella               | Enteroococcus          | Filmbacterin           | Rhodobacter            |
|      | Gallonella              | Enteroococcus          | Filmbacterin           | Rhodopseudomonas       |
|      | Gemmata                | Enteroococcus          | Filmbacterin           | Romboutsia             |
|      | Gemmibacter             | Enteroococcus          | Filmbacterin           | Sandarakininohabudus   |
|      | Granulicellata          | Enteroococcus          | Filmbacterin           | Sediminibacterin       |
|      | Haemophilus             | Enteroococcus          | Filmbacterin           | Singulisphaera         |
|      | Halbacillus             | Enteroococcus          | Filmbacterin           | Sphingobium            |
|      | Halomonas               | Enteroococcus          | Filmbacterin           | Sphingomonas           |
|      | Halophaga               | Enteroococcus          | Filmbacterin           | Sphingopyxis           |
|      | Hungatella              | Enteroococcus          | Filmbacterin           | Sphingorhabdus         |
|      | Hydrotalea              | Enteroococcus          | Filmbacterin           |                     |
| Unit                          | A                              | B                              | C                              | D                              |
|-------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| Metachlamydia                 | Neoehrlichia                    | Mycobacterium                  | Neisseria                       | Nereida                        |
| Methylobacterium              | Nevskia                         | Neisseria                       | Nereida                        | Nitrospira                     |
| Methylophaps                  | Nitrospira                      | Nitrospira                      | Nitrospira                      | Nocardia                       |
| Methyloversatilis             | Niveispirillum                  | Nitrospira                      | Nocardia                       | Novosphingobium                |
| Microbacterium                | Novosphingobium                 | Nitrospira                      | Nocardia                       | Opitutus                       |
| Micrococcus                   | Nitrospira                      | Nitrospira                      | Nocardia                       | Oscillibacter                  |
| Mycobacterium                 | Nitrospira                      | Nitrospira                      | Nocardia                       | Oscillibacter                  |
| Nitrobacteria                 | Paludibaculum                   | Palleronia                      | Pantoea                        | Paracoccus                     |
| Nitrospira                    | Paracaedibacter                 | Paracoccus                      | Paracoccus                     | Paralibacillus                 |
| Novosphingobium              | Paraphilomonas                  | Paracoccus                      | Paralibacillus                 | Paralibacillus                 |
| Opitutus                      | Paraphilomonas                  | Paracoccus                      | Paralibacillus                 | Paralibacillus                 |
| Paludibaculum                 | Paraphilomonas                  | Paracoccus                      | Paralibacillus                 | Paralibacillus                 |
| Pantoae                       | Paraphilomonas                  | Partibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Paracoccus                    | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Pedomicrobium                 | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Pedosphaera                   | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Pelagibacter                  | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Phenyllobacterium             | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Phreatabacter                 | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Pirellula                     | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Planktophila                  | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Prevotella                    | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Propionibrio                  | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Prosthecobacter               | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Protocynobacter               | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Providencia                   | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Pseudolabrys                  | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Pseudomonas                   | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Pseudomonas                   | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Ralstonia                     | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Reyranella                    | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Rhizobacter                   | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Rhizobium                     | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Rhizorhabdus                  | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Rhodanobacter                 | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Rhodobacter                   | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Rhodopseudomonas              | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Rhodovastum                   | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Rickettsia                    | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Romboutsia                    | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Roseburia                     | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Rosemonas                     | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Sandarakinhorbudus            | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Sediminibacterium             | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Selenomonas                   | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Sideroxydans                  | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Sphingobium                   | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Sphingobium                   | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Sphingobium                   | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Spiroplasma                   | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Staphylococcus                | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Streptococcus                 | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Sulfitobacter                 | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Tatslockia                    | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Vampirovibrio                 | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Vampirovibrio                 | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |
| Woesella                      | Paraphilomonas                  | Paralibacillus                   | Paralibacillus                 | Paralibacillus                 |

Total (n) = 122 119 116 51
Streptococcus was present in DUWL water, and other oral bacteria were also found. These oral bacteria are estimated to have entered the water line due to the backflow of water in the ultrasonic scaler. The detection of oral bacteria in DUWL water samples demonstrates the failure of anti-retraction devices in the dental unit. Previous studies have demonstrated the failure of anti-retraction devices through detection of not only oral bacteria but also blood in DUWL water (Coleman et al., 2009; Petti et al., 2013; Walker et al., 2004). This demonstrates the possibility of cross-infection between patients with the use of contaminated DUWL water.

Most bacteria found in DUWL are heterotrophic, with low pathogenicity. However, some opportunistic pathogens that can cause disease in immunocompromised people have also being identified (Atlas et al., 1995; Martin, 1987). In our study, opportunistic pathogens accounted for 7.7% of the total bacteria genera. In other studies, more than 50% of the total genera were opportunistic pathogens (Szymanska and Sitkowska, 2013b). Although the ratios and diversity of opportunistic pathogens found between studies were different, Corynebacterium, Micrococcus, Mycobacterium, Pseudomonas, Sphingomonas, and Legionella are common across DUWLs (Costa et al., 2015; Singh et al., 2003; Szymanska and Sitkowska, 2013b).

High-throughput sequencing, such as the Illumina MiSeq sequencing used in our study, has the disadvantage of expensive analysis costs and short reads that are difficult to identify at the species level. In our study, the number of samples was few and species levels could not be confirmed, so subsequent research examining the bacterial diversity of DUWLs should be carried out with more samples, and further analysis should be performed to identify species accurately. However, high-throughput sequencing can provide more complete information than cultivation techniques because it allows comprehensive detection, including bacteria that cannot be detected by cultivation techniques. In addition, high throughput sequencing has the advantage of being time-efficient because it can sequence hundreds of millions of DNA molecules at a time (Churko et al., 2013).

Although there are very few confirmed cases of opportunistic infections due to the use of contaminated water in dentistry, continuous detection of opportunistic pathogens shows the potential for infection of contaminated DUWL waters. Therefore, periodic DUWL disinfection should be performed to provide safer dental treatment. In particular, the detection of opportunistic pathogens in dental hospitals, which may be visited by immunocompromised patients, may be a more serious problem, so it is necessary to establish measures in relation to this problem in dental hospitals.

A variety of bacteria have been identified from water samples in DUWLs, and there was a difference in bacterial diversity depending on the dental unit. The opportunistic pathogenic bacteria were 7.7% of the total bacteria genera. Our findings show the need for disinfection of DUWLs.

### Table 3

| Group                | Genus (n) | Pathogenic genus                                      |
|----------------------|-----------|-------------------------------------------------------|
| Gram-negative rods   | 167       | Acinetobacter, Alcaligenes, Brevundimonas, Enterobacter, Escherichia, Legionella, Methylobacterium, Pseudomonas, Ralstonia, Sphingomonas |
| Gram-negative cocci  | 24        | -                                                     |
| Gram-negative spiral | 6         | -                                                     |
| Gram-positive rods   | 29        | Bacillus, Corynebacterium, Mycobacterium, Propionibacterium |
| Gram-positive cocci  | 7         | Enterococcus, Micrococcus, Staphylococcus, Streptococcus |
| Total (n)            | 233       | 18                                                    |

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### References

ADA Council on Scientific Affairs (1999) Dental unit waterlines: approaching the year 2000. J. Am. Dent. Assoc., 130, 1653-1664.

Ahn J., Yang L., Paster B.J., Ganly I., Morris L., Pei Z. and Hayes R.B. (2011) Oral microbiome profiles: 16S rRNA pyrosequencing and microarray assay comparison. PLoS One., 6, e22788.

Al-Hiyasat A.S., Ma’ayeh S.Y., Hindiyeh M.Y. and Khader Y.S. (2007) The presence of Pseudomonas aeruginosa in the dental unit waterline systems of teaching clinics. Int. J. Dent. Hyg., 5, 36-44.

Atlas R.M., Williams J.F. and Huntington M.K. (1995) Legionella contamination of dental-unit waters. Appl. Environ. Microbiol., 61, 1208-1213.

Barbeau J., Tanguay R., Faucher E., Avezard C., Trudel L., Cote L. and Prevost A.P. (1996) Multiparametric analysis
of waterline contamination in dental units. Appl. Environ. Microbiol., 62, 3954-3959.

Chun J., Kim K.Y., Lee J.H. and Choi Y. (2010) The analysis of oral microbial communities of wild-type and toll-like receptor 2-deficient mice using a 454 GS FLX Titanium pyrosequencer. BMC Microbiol., 10, 101-2180-2110-2101.

Churko J.M., Mantalas G.L., Snyder M.P. and Wu J.C. (2013) Overview of high throughput sequencing technologies to elucidate molecular pathways in cardiovascular diseases. Circ. Res., 112, 1613-1623.

Coleman D.C., O’Donnell M.J., Shore A.C. and Russell R.J. (2008) Biofilm problems in dental unit water systems and its practical control. J. Appl. Microbiol., 106, 1424-1437.

Costa D., Mercier A., Gravouil K., Lesobre J., Delafont V., Bousseau A., Verdon J. and Imbert C. (2015) Pyrosequencing analysis of bacterial diversity in dental unit waterlines. Water Res., 81, 223-231.

Dutil S., Veillette M., Meriaux A., Lazure L., Barbeau J. and Costa D., Mercier A., Gravouil K., Lesobre J., Delafont V., Bousseau A., Verdon J. and Imbert C. (2015) Pyrosequencing analysis of bacterial diversity in dental unit waterlines. Water Res., 81, 223-231.

Eddy S.R. (2011) Accelerated Profile HMM Searches. PLoS Comp. Biol., 7, e1002195.

Holinger E.P., Ross K.A., Robertson C.E., Stevens M.J., Harris I.K. and Pace N.R. (2007) Microbial diversity of biofilms in dental unit water systems. Appl. Environ. Microbiol., 69, 3412-3420.

Kohn W.G., Harte J.A., Malvitz D.M., Collins A.S., Cleveland J.L., Eklund K.J. and Centers for Disease Control and Prevention (2004) Guidelines for infection control in dental health care settings--2003. J. Am. Dent. Assoc., 135, 33-47.

Martin M.V. (1987) The significance of the bacterial contamination of dental unit water systems. Br. Dent. J., 163, 152-154.

Petti S., Moroni C., Messano G.A. and Polimeni A. (2013) Detection of oral streptococci in dental unit water lines after therapy with air turbine handpiece: biological fluid retraction more frequent than expected. Future Microbiol., 8, 413-421.

Pozza M., Gayoso C., Gomez M.J., Rumbo-Feal S., Tomas M., Aranda J., Fernandez A. and Bou G. (2012) Exploring bacterial diversity in hospital environments by GS-FLX Titanium pyrosequencing. PLoS One., 7, e44105.

Szymanska J. and Sitkowska J. (2013a) Bacterial contamination of dental unit waterlines. Environ. Monit. Assess., 185, 3603-3611.

Szymanska J. and Sitkowska J. (2013b) Opportunistic bacteria in dental unit waterlines: assessment and characteristics. Future Microbiol., 8, 681-689.

Walker J.T., Bradshaw D.J., Bennett A.M., Fulford M.R., Martin M.V. and Marsh P.D. (2000) Microbial biofilm formation and contamination of dental-unit water systems in general dental practice. Appl. Environ. Microbiol., 66, 3363-3367.

Walker J.T., Bradshaw D.J., Finney M., Fulford M.R., Frandsen E., Ostergaard E., Ten Cate J.M., Moorer W.R., Schel A.J., Mavridou A., Kamma J.J., Mandilara G., Stosser L., Kneist S., Araujo R., Contreras N., Goroncy-Bermes P., O’Mullane M.V. and Marsh P.D. (2004) Microbiological evaluation of dental unit water systems in general dental practice in Europe. Eur. J. Oral Sci., 112, 412-418.

Williams J.F., Johnston A.M., Johnson B., Huntington M.K. and Mackenzie C.D. (1993) Microbial contamination of dental unit waterlines: prevalence, intensity and microbiological characteristics. J. Am. Dent. Assoc., 124, 59-65.

Yabune T., Imazato S. and Ebisu S. (2008) Assessment of inhibitory effects of fluoride-coated tubes on biofilm formation by using the in vitro dental unit waterline biofilm model. Appl. Environ. Microbiol., 74, 5958-5964.

Yoon H.Y. and Lee S.Y. (2015) Bacterial contamination of dental unit water systems in a student clinical simulation laboratory of college of dentistry. J. Dent. Hyg. Sci., 15, 232-237.