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Comparison of Bacterial Composition between Human Saliva and Dental Unit Water System

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The bacterial compositions between the dental unit water system and human saliva were characterized and compared by direct sequence analysis of 16S rDNA clone libraries. Based on the species richness estimation, bacterial diversity in the dental unit water system (DUW) was more diverse than that of the human saliva (HS). The Chao1 estimates of species richness in HS and DUW samples were 12.0 and 72.4, respectively. The total numbers of OTUs observed in the combined libraries accounted for 83% (HS) and 59% (DUW) of the Chao1 diversity estimate as defined at the 80% similarity threshold. Based on the sequence analysis, the phylum *Proteobacteria* was the major group in both clone libraries at phylum level. DUW clone library contained 80.0% *Proteobacteria*, 8.0% *Bacteroides*, 4.0% *Nitrospira*, 4.0% *Firmicutes*, 2.0% *Planctomycetes* and 2.0% *Acidobacteria*. On the other hand, human saliva (HS) clone library contained 55.5% *Proteobacteria*, 36.1% *Firmicutes* and 8.4% *Bacteroides*. The majority of bacteria identified belonged to phylum *Proteobacteria* in both samples. In dental unit water system (DUW), *Alphaproteobacteria* was detected as the major group. There was no evidence of the bacterial contamination due to a dental treatment. Most sequences were related to microorganisms derived from biofilm in oligotrophic environments.

Keywords: human saliva, dental unit water, 16S rDNA clone, species richness

Microbial infection control is one of the most important issues in medical facilities such as dental offices and hospitals (Molinari, 2003). A variety of disposable medical supplies were developed and are used to prevent the cross-infection between the patients, but dental clinics are exposed to relatively higher infection risk than other medical facilities. Moreover, dental equipments which directly contact surgical wounds are frequently exposed to the cross infection risk (Williams *et al.*, 1996). For effective infection control, every possible source of contamination should be eliminated by proper treatment such as sterilization, disinfection, and use of disposable supply before, during and after dental intervention. However, the dental unit, which has a waterline as essential system, is exposed without any defensive barrier to the bacterial infection from the water system (U.S. Environmental Protection Agency, 1992). If bacterial contamination occurred in the water reservoir, it could be a potential health or fatal infection risk. Recently, dental units are maintained by using an integrated disinfection system or periodic managements and cleaning. However, the investigation of the microbial community composition will be required to maintain effective disinfection management. Dental aerosol and irrigate water were revealed as major sources of the bacterial infection, and microorganisms in dental unit water (DUW) biofilm are predominantly derived from the incoming water (Walker *et al.*, 2000). Bacteria shed from the biofilm during use maintain the bio-burden of planktonic organisms detected in DUW (Pankhurst *et al.*, 1998). The presence of the biofilms also can act as a reservoir for potential pathogens and increases the health risk to the immunocompromised patients (Martin, 1987). Although many studies have investigated the bacterial diversity in the water delivered from dental units (Singh *et al.*, 2003), only a few have archived bacterial identification. Also, conventional culture technique does not provide a representative profile of the microbial composition, and thus we would like to provide a more complete representation of the microbial community by using 16S rRNA gene sequence as a phylogenetic marker.

The primary aim of this study was to examine the microbial community composition in the dental unit water and human oral environment with culture-independent technique. As a result of this research, the effective treatment for microbial disinfection of the dental unit waterline could be provided and the importance of periodical management in dental unit water system should be considered.

Materials and Methods

Sample collection and genomic DNA extraction

Saliva sample (5 ml) of a healthy and young female was collected using sterilized 10 ml plastic tube at the dental unit in Dankook University during the regular check of oral health. Two hours before saliva sampling, the subjects brushed and had no food and drinks. This subject had no periodontal problems and no record of antibiotic use during the previous 3 months. Two liters of dental unit water was collected from dental unit at Dankook University, and filtered by using Sterivex GV filter unit (Millipore, USA).

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Genomic DNA was prepared with enzymatic, chemical and physical process. Five hundred ul of lysis buffer (10 mM Tris·Cl, 100 mM EDTA, 15 mg/ml lysozyme, pH 8.0) was added to the filter unit and the filter unit was incubated at 37°C for 60 min with occasional agitation. The mixture was treated with 50 μg/ml of Proteinase K at 55°C for 30 min and then 1 % sodium dodecyl sulfate was added as a final concentration. Three cycles of freezing (-70°C) and thawing (65°C) were conducted. The treated mixture was transferred into new centrifuge tube and an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added. The mixture was centrifuged at 6,000×g for 10 min. Then, the aqueous phase was collected and mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 6,000×g for 10 min. Liquid phase was collected and an equal volume of ice-cold isopropanol was added to precipitate the nucleic acids. The tube was stored overnight at -20°C. Nucleic acids were pelleted by centrifuging at 10,000×g for 10 min, and DNA was washed with 70% ethanol and resuspended with 500 μl TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 8.0).

PCR amplification and cloning

Bacterial 16S rRNA gene was amplified from genomic DNA by PCR with primers designed to anneal to conserved regions in 3' and 5' of bacterial 16S rRNA genes. The forward primer (bacterial 27F; 5'-AGAGTTTGATCMTG GCTCAG-3') corresponded to positions 8 to 27 of *Escherichia coli* 16S rRNA gene, and the reverse primer (Universal 1492R; 5'-GGYTACCTTGTTACGACTT-3') corresponded to the complement of positions 1492 to 1510 (Lane, 1991). The 50 μl reaction mixture contained the following final concentrations or total amount: 1 μl template DNA (50 ng/μl), 5 μl of 10× reaction buffer (100 mM Tris·Cl, 500 mM KCl, pH 8.5), 1.5 mM $MgCl_2$, 5 μl of dNTPs (2.5 mM each), 10 pmoles of each primer, and 1 unit of *Taq* DNA polymerase. PCR Amplification was carried out with thermocycler (Perkin-Elmer, USA). All reagents were mixed and then heated to 94°C for 4 min. Thirty cycles of the PCR were then run at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min followed by 72°C for 10 min. The purified PCR products were then cloned into pGEM-T vector (Promega, USA) which is constructed with a 3'-terminal thymidine on each end of a blunt-end digestion product, thus improving the efficiency of ligation of PCR products into the vector by taking advantage of the non-template-dependent addition of a single deoxyadenosine to the 3' end of PCR product by *Taq* DNA polymerase.

Sequence analysis

Plasmid DNAs were isolated from randomly picked bacterial colonies by Plasmid miniprep kit (Bio-rad, USA) and were sequenced with M13R primer. All sequences were about 600 bases long, checked for chimeric artifacts by CHECK_CHIMERA program and compared to similar rDNA sequences retrieved from SSU database of Ribosomal Database Project (RDP), as well as GenBank database. Partial sequences were determined for each OTU identified in the 16S rRNA gene library, and these were submitted to RDP Classifier using default parameters (confidence threshold, 80%) to determine phylogenetic affiliations of the respective bacteria, which were defined according to Ribosomal Database Project Classifier (http://rdp.cme.msu.edu/classifier.jsp). Based on the result of RDP Classifier, species richness and Chao1 estimation were calculated by EstimateS program (Version 7.5, http://viceroy.eeb.uconn.edu/EstimateS) (Colwell, 2005). Pairwise sequence alignment was performed with the nearest neighbor sequence and manually curated in PHYDIT program (Chun *et al.*, 2000). All reference sequences of aligned 16S rDNA were obtained from RDP database. Taxonomic assignments were done by comparing the clone sequences with the non-redundant nucleotide database in RDP database by RDP classifier (Cole *et al.*, 2005). A Jukes-Cantor corrected distance matrix was calculated by DNADIST (PHYLIP package version 3.66). The Neighbor-joining tree was constructed by NEIGHBOR (PHYLIP package version 3.66, http://evolution.genetics.washington.edu/phylip) and visualized by using TreeView program (Version 1.6.1, taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Nucleotide sequence accession numbers

The partial sequences of 16S rDNA were deposited to GenBank database under the accession numbers listed EF095773 to EF095954.

Results and Discussion

Comparison of diversity indices

OTU (operational taxonomy unit) defined as classifying the sequence with genus level by RDP Classifier and diversity indices were calculated with the number of OTUs defined (Cole *et al.*, 2005). Based on the result of diversity indices, the bacterial community in dental unit water (DUW) sample was more diverse and more evenly distributed than that of human saliva (HS) sample (Table 1). Chao1 estimation as non-parametric estimator is a useful method to predict the true diversity of the total population (Hughes *et al.*, 2001). Also rarefaction analysis is more a valuable way to compare the relative diversity of communities (Hughes *et al.*, 2001). As shown in Fig. 1, HS sample had a relatively low level of phylotype richness, whereas DUW sample had high richness values. The Chao1 estimates of species richness in HS and DUW samples were 12.0 and 72.4, respectively. The total numbers of OTUs observed in the combined libraries accounted for 83% (HS) and 59% (DUW) of the Chao1 diversity estimate as defined at the 80% similarity threshold. This indicates that the HS and DUW clone libraries were well represented for the whole bacterial diversity in that system (Table 1). Based on the sequence analysis, the phylum *Proteobacteria* was the major group in both clone libraries at phylum level. Dental unit water (DUW) clone library contained 80.0% *Proteobacteria*, 8.0% *Bacteroides*, 4.0% *Nitrospira*, 4.0% *Firmicutes*, 2.0% *Planctomycetes* and 2.0% *Acidobacteria*. On the other hand, human saliva (HS) clone library contained 55.5% *Proteobacteria*, 36.1% *Firmicutes* and 8.4% *Bacteroides*.

Microbial diversity in dental unit water system

In DUW clone library, 81 sequences were closely aligned with uncultured bacterial 16S rDNA genes in BLAST result

Table 1. Diversity indices and bacterial composition in the samples

	HS	DUW
No. of OTUs	10	43
Individual	82	100
Shannon Weaver diversity	1.90	3.41
Evenness	0.83	0.91
Chao1 estimation	12.0±3.0	72.4±12.9
Richness	4.7	20.5
Coverage[a]	83%	59%
Class *Alphaproteobacteria*	ND[b]	37.0
Class *Betaproteobacteria*	14.5	24.0
Class *Gammaproteobacteria*	41.0	12.0
Class *Deltaproteobacteria*	ND	7.0
Phylum *Bacteroidetes*	8.4	8.0
Phylum *Nitrospira*	ND	4.0
Phylum *Firmicutes*	36.1	4.0
Phylum *Planctomycetes*	ND	2.0
Phylum *Acidobacteria*	ND	2.0

[a] Percentage of coverage: percentage of observed number of OTU divided by Chao1 estimate.
[b] not detected.

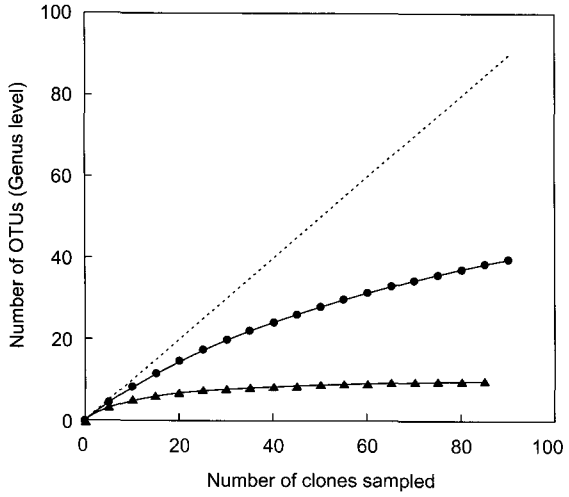

Fig. 1. Rarefaction curves for clone libraries of 16S rDNA gene in dental unit water system (DUW, circle) and human saliva (HS, triangle)

(Fig. 2). Most sequences were closely aligned with a variety of *Alphaproteobacteria* taxa (37.0%), including *Sphingomonas*, *Azorihizobium*, *Hypomicrobium*, *Acetobacter*, *Afipia* and *Rhodopseudomonas* species. Second major sequences were affiliated with *Betaproteobacteria* (24.0%). The majority of 12 sequences (DUW18, 19 etc.) in *Betaproteobacteria* were closely related to uncultured bacterium DSSD10 (AY328709) and DSSD46 (AY328744), which were found in chloraminated Cincinnati distribution system simulator discharge water (Williams *et al.*, 2004). Third major sequences were aligned with *Gammaproteobacteria* (12.0%), including *Acinetobacter*, *Hydrocarboniphaga*, *Lysobacter*, *Pseudoxanthomonas*, and *Rickettsiella*.

A previous study of the bacterial distribution in drinking water distribution system simulator (DSS) demonstrated that *Alphaproteobacteria* was the most abundant group in drinking water system, suggesting that these organisms are well suited to survive following exposure to the disinfectants (chlorine and monochloramine) in potable water supplies (Williams *et al.*, 2004). Bacteria are not much exposed to the disinfectant in biofilms of water distribution system which is capable of protecting the bacterial consortia from the chlorination, though the free-living bacteria are much more sensitive to chlorine disinfectant exposure. According to the result of Singh *et al.* (2003), the major group belonged to the phylum *Proteobacteria* (55%), containing 23% *Alphaproteobacteria*, 11% *Betaproteobacteria*, 15% *Gammaproteobacteria* and 7% *Deltaproteobacteria*. Another 45% of biofilm bacteria were comprised of *Leptospira* (20%), *Bacillus* (7%), *Planctomyces* (2%) and so on. Some organisms found in dental unit water system may have a potential pathogenic resource for the immunocompromised individuals. *Sphingomonas* sp. strains secrete viscous polysaccharide, which aid to adhere to the surface of pipelines. Their presence in dental unit water is a main concern, since microorganisms may engender health risk to patients and dental personnel during dental treatment. Our study showed the presence of these eubacterial groups and predominance of *Proteobacteria* in DUW clone library. Therefore, we could presume that DUW clones were mainly derived from the biofilm of DUW.

Microbial diversity in human saliva

In HS clone library, 34 sequences were aligned with *Gammaproteobacteria* as the dominant group. In contrast 37% of DUW clones were classified in *Alphaproteobacteria* as a dominant group (Fig. 2). The majority of 25 sequences in *Gammaproteobacteria* were closely related to *Haemophilus* species, which is an intrinsic pathogen that causes endocarditis. Second major sequences (36.1%) were affiliated with *Firmicutes*, belonging to the *Streptococcus*, *Veillonella*, *Granulicatella*, and *Solobacterium*. Third major sequences (14.5%) were affiliated with *Betaproteobacteria* and all sequences were matched to *Neisseria* species, a pathogen for meningitis.

Ten genera were classified in HS clone library, including *Haemophilus* (30.5%), *Streptococcus* (23.2%), *Neisseria* (14.6%), *Prevotella* (8.5%), *Veillonella* (7.3%), *Mannheimia* (6.1%), *Granulicatella* (4.9%), *Actinobacillus* (2.4%), *Lonepinella* (1.2%), and *Solobacterium* (1.2%). All major genera are often detected in human mouth as a normal oral microflora. *Haemophilus paraphrophilus* is a slow-glowing and fastidious commensal microorganism of the oral cavity and pharynx. It can cause subacute bacterial endocarditis and brain abscess among other conditions. *Streptococcus salivarius* is a predominant member of the normal oral flora of healthy humans (Doel *et al.*, 2005). When oral pH begins to decrease and maintains acidic condition by anaerobic fermentor, acidogenic strains of *Streptococcus mutans* and *Lactobacillus rhamnosus* increased, whereas this decrease in pH was accompanied by a fall in the proportion of acid sensitive species such as *Neisseria subflava, A. naeslundii, S. oralis, S. gordonii, Prevotella nigrescens,* and *Fusobacterium nucleatum* (Bradshaw and Marsh,

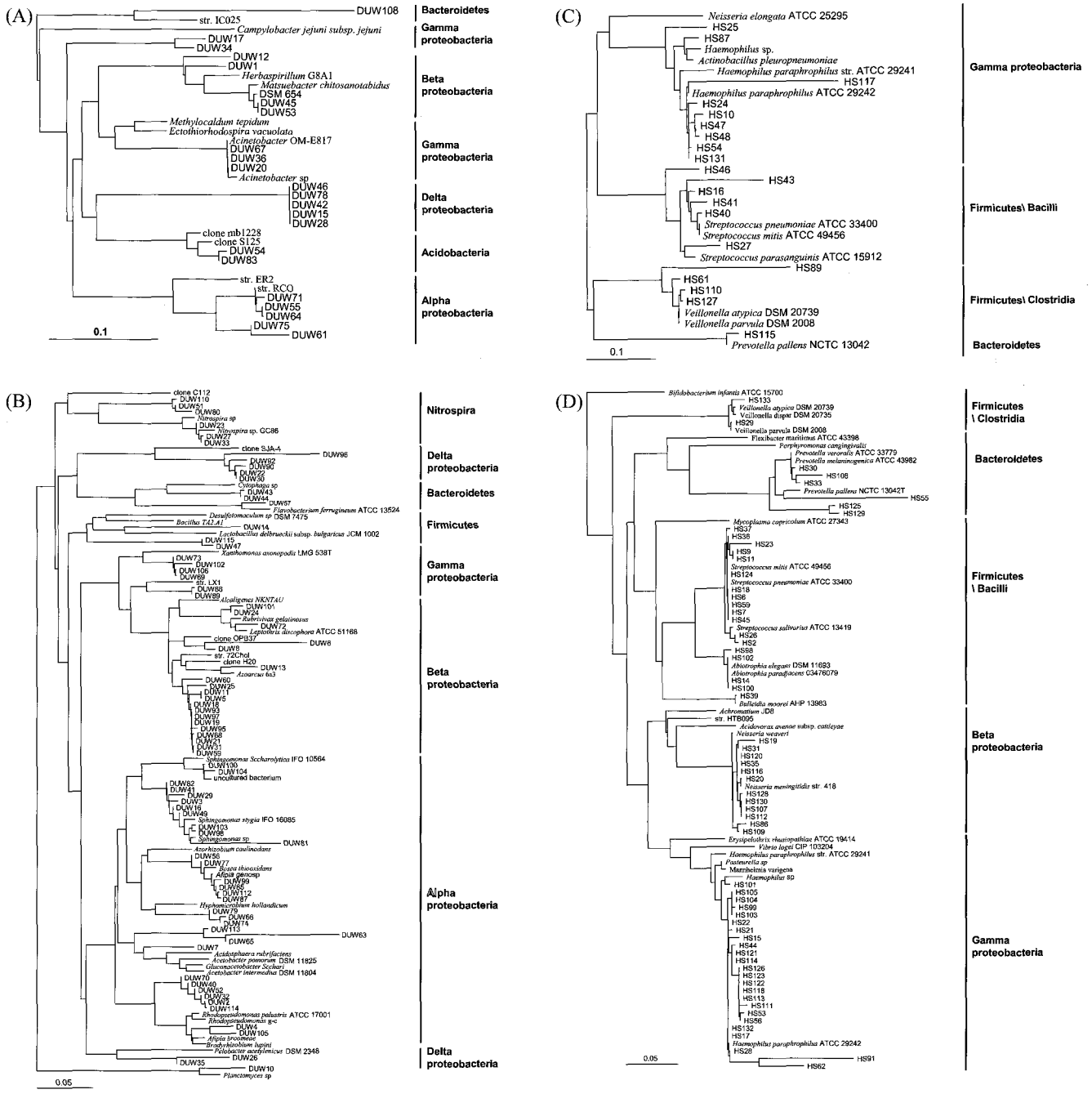

Fig. 2. Phylogenetic tree constructed with Neighbor joining analysis showing the affiliations of the partial 16S rRNA sequences, using nucleotides (A) 30~253 (DUW), (B) 1205~1455 (DUW), (C) 40~440 (HS), and (D) 1115~1445 (HS) corresponding to *E. coli* 16S rRNA numbering and alignment, determined from 16S rDNA clone libraries collected from dental unit water (DUW) and human saliva (HS). The closest known sequence from GenBank is included as a reference for each sequence

1998). Although six clones (7%) were aligned with *Veillonella dispar*, which is the most numerous organism at lower pH (5.0-4.5), the sequences related with *Streptococcus, Neisseria, Prevotella,* and *Veillonella* were detected in the sample so that we can presume it was normal condition. As a result of HS clone analysis, the microbial distribution of the clinical subject was a normal condition and there was no evidence of caries and periodontal disease.

The aim of this study was to examine the relationship between human saliva and dental unit water by using 16S rDNA analysis. We expected that some microorganisms may exist in both samples or contaminate from the patients by suck-back effect, but there was no evidence of the bacterial contamination due to the dental treatment. Most sequences were related to microorganisms derived from biofilm in oligotrophic environments. Nevertheless, dental unit water system should

be regularly maintained, because the water introduction into patients of such high levels of microorganisms must be of concern and always has a potential risk for immunocompromised individuals.

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