New Microbiologica, 39, 2, 143-145, 2016, ISN 1121-7138

SHORT COMMUNICATION

Molecular analysis for bacterial contamination in dental unit water lines

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SUMMARY

Bacterial contamination in dental unit waterlines (DUWLs) was evaluated by molecular techniques in addition to the conventional culture method. Water samples (n=8) from DUWLs were investigated for heterotrophic bacteria by culture method using R2A agar. The selected bacterial antibiotic-resistance genes and *Legionella* species-specific 16SrDNA were identified by PCR. The profiles of bacterial contamination in DUWLs were further identified by PCR-DGGE. In this study, no antibiotic-resistant or *Legionella* genes were detected. Polycyclic aromatic hydrocarbon-degrading bacterium, *Novosphingobium* sp. was the most prevalent in DUWLs. Conventional PCR and PCR-DGGE were shown to be potentially useful for monitoring of bacterial contamination in DUWLs.

Received September 19, 2015 *Accepted* March 18, 2016

Dental chair units are equipped with a narrow-bore, flexible, plastic tubing called dental unit waterlines (DUWLs) that supply water to all dental instruments (air/water syringe, turbine, rinsing equipment) (O'Donnell *et al.*, 2011). Bacterial contamination is often observed in the complex dental unit. Therefore, biofilms containing multiple bacterial species easily form within DUWLs (Cobb *et al.*, 2002). Bacterial numbers per ml in DUWLs can reach several million overnight (Depaola *et al.*, 2002). Heavy microbial contamination of DUWLs is thought to be the result of biofilm formation within small-bore plastic tubes used to supply water to dental equipment (Pankhurst *et al.*, 1998). Opportunistic bacteria such as *Legionella pneumophila* and *Pseudomonas aeruginosa* have previously been revealed in biofilms within DUWLs (Petti *et al.*, 2004). In addition, *Staphylococcus* sp. isolated from DUWLs are resistant to several antibiotics (Lancellotti *et al.*, 2007; Güngör *et al.*, 2014). Thus, bacterial contamination in DUWLs is a recent growing concern in dentistry. Several human pathogens in the environment can change to a "viable but non-culturable" state and are not detectable by culture methods (Oliver 2005; Su *et al.*, 2013). The aim of this study was to monitor bacterial contamination in DUWLs using molecular techniques based on bacterial 16S ribosomal RNA gene (16SrDNA) and antibiotic-resistance genes.

In this study, bacterial contamination in the 8 DUWLs located at a dental hospital in Japan were investigated by molecular techniques in addition to the conventional culture method. Water samples were collected at the end of the working day, after 3.5 days, and at the beginning of the working day before use. After appropriate dilutions, the water samples were plated on R2A agar (DAIGO®, Nihon Pharmaceutical Co. Ltd, Tokyo, Japan), selective for heterotrophic bacteria and incubated at 25°C for 7 days, to obtain total bacterial counts in terms of colony forming units (CFU) per ml. Abundant white and off-white colonies were observed on the R2A agar plates, from each DUWLs sample. The average bacterial count at end of the working day, for each DUWL water sample was as follows:

1) 390 CFU/ml,
2) 3,020 CFU/ml,
3) 3,780 CFU/ml,
4) 552 CFU/ml,
5) 435 CFU/ml,
6) 175 CFU/ml,
7) 1,245 CFU/ml,
8) 3,320 CFU/ml.

Thus, only 3 chair units were found to meet the Centers for Disease Control and Prevention (CDC) recommended water quality standard in terms of the maximum permissible level of contamination by heterotrophic bacteria in DUWL water at the end of the working day, at 500 CFU/ml (Kohn *et al.*, 2004). On the other hand, the average bacterial count for each DUWL water sample at beginning of the working day was as follows:

1) 6,090 CFU/ml,
2) 6,325 CFU/ml,
3) 3,925 CFU/ml,
4) 4,915 CFU/ml,
5) 5,122 CFU/ml,
6) 795 CFU/ml,
7) 2,810 CFU/ml,
8) 6,345 CFU/ml.

Key words:
Bacterial contamination, Dental unit water line, Molecular analysis, PCR-DGGE.

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Additionally, after 3 and a half days of not being in use, the bacterial count in each DUWL was highly increased. Thus, the quality of water from the DUWLs sampled did not fulfilling the water standards criteria set by the CDC. Colonies formed were used for 16SrDNA analysis. As a result, *Novosphingobium* sp. and *Sphingomonadaceae* sp. were identified as predominant contaminants in DUWL samples based on 16SrDNA sequencing of isolates on R2A agar.

Several previous reports have indicated that flushing with water for several minutes at the beginning of the working day effectively reduces the bacterial load in DUWLs (Pankhurst *et al.,* 1998; Cobb *et al.,* 2002; Watanabe *et al.,* 2008). However, biofilm formation within the DUWLs presents a challenge for complete elimination of bacteria by flushing with water (O'Donnell *et al.,* 2011). O'Donnell *et al.* concluded that chemical DUWL cleaning, dental chair unit design, and development of automated DUWL cleaning control systems provide effective long-term solutions to DUWL maintenance. Water from DUWLs and hospital wastewaters is not sterile and contains high numbers of bacteria. Several studies have reported the presence of opportunistic pathogens and antibiotic-resistant bacteria in DUWLs (Petti *et al.,* 2004; Al-Hiyasat *et al.,* 2007; Máayeh *et al.,* 2008; Arvand *et al.,* 2013) and hospital wastewaters (Schwartz *et al.,* 2003; Yomoda *et al.,* 2003; Thompson *et al.,* 2013).

In this study, contamination by antibiotic-resistant pathogens and *Legionella* species in DUWLs was further examined by conventional polymerase chain reaction (PCR) method. Each 50-mL water sample collected at the beginning of the working day was centrifuged at 10,000×*g* for 30 min at 4°C. The supernatant was discarded and the pellet was suspended in 200 µL InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA). Bacterial DNA was extracted according to the manufacturer's instructions and used for PCR. Antibiotic-resistant pathogens, namely methicillin-resistant *S. aureus* (MRSA), multi-drug resistant *Pseudomonas*, extended-spectrum beta-lactamase (ESBL) producing bacteria, and vancomycin-resistant *Enterococci* were identified by PCR, by amplification of respective bacterial genes, *mecA*, *bla*IMP, *bla*VIM, *bla*TEM, *vanA*, and *vanB*, as previously described (Murakami *et al.,* 1991; Kariyama *et al.,* 2000; Heneriques *et al.* 2006). Contamination by *Legionella* sp. was also investigated using PCR for specific 16SrDNA (Yamamoto *et al.,* 1993). Neither bacterial antibiotic-resistance genes nor *Legionella*-specific 16SrDNA were detected in any of the DUWL samples analyzed by PCR.

Moreover, the profiles of bacterial contamination in DUWLs were evaluated by PCR-denaturing gradient gel electrophoresis (DGGE) in this study. The PCR-DGGE method has become one of the new analytical tools for microflora (Fujimoto *et al.* 2003). A forward primer with a 40-bp GC-rich sequence added to the 5′-end (5′-CGCCCGCCG-CGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-CCTAC-GGGAGGCAGCAG-3′) and a reverse primer (5′-CCGT-CAATTCCTTTRAGTTT-3′) were used to amplify by PCR, approximately 585-bp 16SrDNA fragments corresponding to nucleotide positions from 341 to 926 in the genome sequence of *Escherichia coli*. PCR was performed using EX-Taq® kit (TaKaRa Shuzo Ltd., Otsu, Japan) as previously described (Fujimoto *et al.,* 2003). DCode Universal Mutation Detection System (Bio-Rad Lab.) was used for sequence-specific separation of the PCR products on 6% (w/v) polyacrylamide gel containing a linear gradient denaturant, ranging from 20% to 50%. The gel was run for 4 h at 200 V in 0.5X Tris-acetate-EDTA (TAE) buffer (1X TAE buffer contains 0.04 mol/l Tris base, 0.02 mol/l acetic acid, and 1.0 mmol/l EDTA, pH 8.5) at a constant temperature of 60°C. After staining with ethidium bromide, the gel was viewed under UV transillumination. The dominant 16SrDNA bands on the denaturing gradient gel were excised, purified, and re-amplified using PCR as previously described (Fujimoto *et al.,* 2003). The re-amplified DNA fragments were cloned into a vector (TOPO TA Cloning kit®, Invitrogen, Carlsbad, CA, USA), and the DNA inserts were sequenced using a kit (ABI Prism BigDye® terminator v1.1 cycle sequencing ready reaction kit, Perkin Elmer, Foster City, CA, USA) and an automated DNA sequencer (ABI PRISM 3100, Perkin Elmer). The sequence data were subjected to the BLAST sequence homology search program at GenBank and analyzed. Bacterial species were identified at similarity values above 99%.

PCR-DGGE analysis revealed that polycyclic aromatic hydrocarbon-degrading bacterium, *Novosphingobium* sp., were the most prevalent and dominant contaminating bacteria in all DUWL samples tested. *Dechloromonas* sp., *Blastobacter* sp. and *Sphingomonadaceae* sp. were also identified as other, minor contaminants (*Figure 1, Table 1*). *Novosphingobium* sp. and *Sphingomonadaceae* sp. were identified in all DUWL water samples, by both, culture method and PCR-DGGE analysis. Additionally, PCR-DGGE analysis revealed the profiles of bacterial contamination in the DUWLs. Moreover, polycyclic aromatic hydrocarbon-degrading bacterium, *Novosphingobium* sp. was the most prevalent and dominant, whereas, *Dechloromonas* sp., *Blastobacter* sp., and *Sphingomonadaceae* sp.

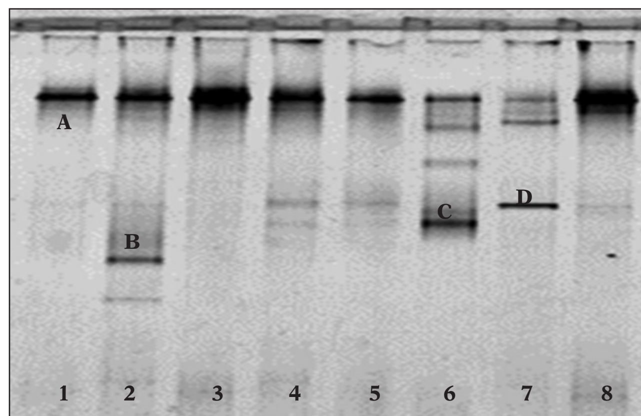

Figure 1 - *DGGE profiles of amplified 16SrDNA from 8 DUWL water samples. Lanes 1 to 8 correspond to the sample number of DUWLs described in the text. The 4 intense bands (A-D) obtained were cloned and sequenced. The bacterial species identified by sequencing are shown in Table 1.*

Table 1 - *Bacterial species identified from DGGE major bands.*

Bands in Fig. 1	*Identified species (the number of clones isolated)*
A	*Novosphingobium* sp. (8)
B	*Sphingomonadaceae* sp. (1)
C	*Blastobacter* sp. (1)
D	*Dechloromonas* sp. (1)

were identified as minor contaminating bacteria in the DUWL samples tested in this study. The pathogenicity of *Novosphingobium* sp., *Dechloromonas* sp., *Blastobacter* sp. and *Sphingomonadaceae* sp. in healthy and immunocompromised subjects is still unknown (Rutebemberwa *et al*., 2014). These bacteria might be useful for one of indicators of water quality in DUWL.

Hospitalized patients with infectious diseases and the elderly requiring nursing-care are immunocompromised and more likely to visit dental clinics for dental care and treatment, where both patients and clinic staff could be exposed to microorganisms in DUWL output water and contaminated aerosols. Although the case of infection resulting from exposure to water from the contaminated DUWL is limited (Ricci *et al*., 2012), it is necessary to monitor the level of bacterial contamination in DUWLs and maintain the water quality.

In conclusion, PCR-DGGE was shown to be a potentially useful molecular analysis tool to monitor DUWL bacterial contamination. Conventional PCR for antibiotic-resistance genes and *Legionella* sp. 16SrDNA could also be used for rapid monitoring and tracing of the source of nosocomial infections via DUWLs.

Acknowledgements

This work was supported by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) (KAKENHI Grant Number 26463163). The authors declare no conflicts of interest in the publication of this study.

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