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High throughput sequencing-based analysis of microbial diversity in dental unit waterlines supports the importance of providing safe water for clinical use

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ABSTRACT

This study aims to explore the water quality of dental unit waterlines (DUWLs) and the diversity of microbial communities in DUWLs. Water samples from 33 dental chair units (DCUs) were collected, diluted and then spread on sterilized R2A plate for incubation. Subsequently, the microbial colony-forming units per milliliter (CFU/ml) were recorded by an automatic colony analyzer. Total DNA extracted from the rest of the samples was tested on the Illumina MiSeq PE300 platform. T-test and Kruskal–Wallis rank test were adopted for statistical analysis. Significance was assumed at a $P < 0.05$. After incubation, the average total microbial count was 21,413.13 ± 17,861.00 CFU/ml. High-throughput sequencing revealed 10 bacterial phyla, including 9 identified and 1 unclassified phyla. Totally 63 sequences were identified at the genus level, including 42 genera, 3 tentative species and 18 unclassified genera. In addition, 7 potential human pathogenic bacteria were detected. In summary, department, brand and service life of DCUs do not influence the water quality of DUWLs significantly. The diversity of microbial communities in DUWLs is abundant and includes both pathogenic and some unknown bacteria.

Introduction

As important parts of dental chair unit (DCU), dental unit waterlines (DUWLs) provide water to cool and irrigate both the instruments and tooth surfaces during dental treatment, which leads to the fact that the water quality is crucial and may relate to cross-infection during the treatment. As early as 1963, a British dentist Blake first reported microbial contamination in DUWLs [1]. Many subsequent studies have found different opportunistic pathogens in DUWLs, including *Legionella species, Staphylococcus, Streptococcus, Mycobacterium tuberculosis, Pseudomonas aeruginosa* and *fungi* [2–6]. Microbial contamination in DUWL can happen in many ways: (1) Anti-retraction valves failure in high-speed handpieces or three-in-one air/water syringes could lead to fluids backflow from the oral cavity into DUWLs; (2) Narrow bore of waterlines would cause low water velocity, which promotes the deposition of microorganisms on the luminal surface of DUWLs [7]; (3) Water stagnation in DUWLs encourages the formation and growth of biofilm when DCUs are not used; (4) Heating the DCU output water may selectively aggravate the growth of particular bacterial species [8,9]; (5) The quality of source water, which is usually municipal water, can also cause the microbial contamination. Opportunistic pathogens can seed on the internal wall of tubes or drop from the biofilms and then enter the oral cavity directly via high-speed handpieces or three-in-one air/water syringes. This can increase the risk of cross-infection among patients and dental healthcare personnel [10], therefore the water quality of DUWLs must be closely monitored.

Although bacterial cultivation is the most common method to monitor the quality of DCUs output water, metagenomics has developed rapidly since the beginning of the 21st century because it can comprehensively reveal the diversity of microbial communities in the ways that cultivation could not [11]. The Illumina MiSeq PE300 sequencing platform used in this study is a high-throughput sequencing platform based on bacterial 16S rDNA that enables the production of highly accurate sequencing data. Previous studies of environmental monitor demonstrated that the platform can

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Table 1
Departments, brands and service lives of the 33 DCUs.

Department	Brand	N	Onset of use (year)				
			2002	2003	2008	2011	2013
Periodontics	SIRONA C8+	10	0	5	2	0	3
Endodontics	SIRONA C8+	15	4	0	4	7	0
	A-dec 8000	8	0	0	0	8	0

provide new insights into studying the diversity of microbial communities in the environment [12,13].

In this study, we used R2A culture method to identify the microbial contamination in DUWLs. In addition, Illumina MiSeq Sequencing techniques were adopted to further elucidate the microbial communities especially for some pathogens that difficult to cultivate and the diversity of microbial communities in DUWLs. The results would help us to assess the potential risks of microbial contamination in DUWLs, formulate output water standards, propose reasonable strategies to control biofilm and support the fact that providing safe water for clinical use is of vital importance in China.

Materials and methods

Water samples were collected from the Endodontics and Periodontics Departments in the Affiliated Hospital of Stomatology, Nanjing Medical University. All recruited DCUs were required to conduct common dental treatments within 3 months and have no repair record within 1 month before sampling (Table 1). More importantly, DCUs that treated patients with infectious diseases (e.g. acquired immune deficiency syndrome (AIDS), hepatitis B) within 3 days of sampling were excluded. All the recruited DCUs used filtered tap water as source water, and the water quality met the criterion of the "National Standards for Drinking Water Quality", which indicates that the amount of bacteria in the water must be less than 100 colony-forming units per milliliter (CFU/ml). Before sampling day, all recruited DCUs were in normal clinical use and turned off in the end of the day.

Water sample collection

Before patients arrived in the sampling day, a 200-ml water sample was collected from each DCU using a three-in-one air/water syringe. The water samples were put into sterile bottles after the DCUs were turned on and flushed for 30 s as clinical application and then transported to the microbiology laboratory as soon as possible.

Bacteria culture and analysis

The water samples were mixed for 15 s and then diluted 10-fold separately with sterilized ddH_2O. Next, 100 μL of each prepared solution was placed on a sterilized R2A plate (BD, Franklin Lakes, New Jersey, USA) and spread with a triangular glass rod coater immediately on a Clean Bench (Air Tech, Suzhou, Jiangsu, China). When the solution was absorbed completely, the R2A plates were inverted and incubated at 37 °C for six days. Each water sample was plated in triplicate, and two blank R2A plates from the same batch were used as blank controls. After incubation, the number of microbial CFU/ml per plate was quantified using an automatic colony analyzer (Interscience Scan® 200, du Bois des Arpents, St Nom la Bretêche, France).

For quantitative study, T-tests and analyses of variance (ANOVA) were applied to compare bacterial contamination values. Kruskal-Wallis rank test was used to analyze the quantitative data that did not meet the homogeneity of variance. Significance was assumed at a $P < 0.05$.

DNA extraction

The remaining samples were centrifuged at 8000 *g* for 3 min. The sediment was collected and suspended in 500 μl of ddH_2O. Bacterial genome DNA extraction was accomplished using a TaKaRa MiniBEST Bacteria genomic DNA Extraction Kit Ver.3.0 (Takara, Dalian, China) following the manufacturer's protocol and stored at −20 °C until use for molecular applications.

PCR amplification and high-throughput sequencing

The DNA extracts were sent to a commercial company (Majorbio, Shanghai, China) for further PCR amplification and Illumina MiSeq sequencing. Specific steps were presented as follows: the extracted genomic DNA was amplified with a set of primers targeting the V4 and V5 hypervariable regions of bacterial 16S rRNA genes. The primers were 515F (5′-TGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′). PCR amplification was performed in triplicate using a 20 μl reaction mixture containing 4 μl of 10 × Buffer, 1.6 μl of dNTPs (2.5 mM), 0.8 μl of each primer (μM), 0.2 μl of rTaq DNA polymerase (TaKaRa, Dalian, China), 0.2 μl of bovine serum albumin (BSA), and 10 ng of template DNA. Thermal cycling conditions were presented as follows: an initial denaturation at 95 °C for 3 min, 27 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, with a final extension at 72 °C for 10 min. Mixtures of PCR products from the same sample were examined on agarose gel (2% in Tris/borate/EDTA buffer). After illuminating the gel under long-wavelength UV light, the band of interest was cut and purified using the AxyPrep DNA Gel Extraction Kit (Axygen, CA, USA). Amplicons of each sample were used for sequencing on the Illumina MiSeq PE300 platform.

Statistical analysis

After the raw sequences were obtained, paired-end reads with no less than 20 bases were merged from overlapping paired-end reads using the FLASH program, and low-quality fragments, barcodes and primers were removed for quality control requirement.

The sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity level in the research program. Based on optimized OTUs, the Alpha-diversity indices (including Chao 1, Ace, Shannon, and Simpson indices) were calculated and compared by two-tailed T-test to determine the significance of the differences in bacteria diversity between the Endodontics and Periodontics Departments (at a value $P < 0.05$). Total 25,000 sequences were randomly selected to generate rarefaction curves using the Mothur program. The taxonomic annotation of representative OTUs at a similarity level of 97% was matched with SILVA sequences by RDP's Classifier at a 70% threshold. Principal Co-ordinates Analysis (PCoA) and hierarchical clustering analysis were performed to examine the interrelationships of the bacterial communities between the two departments.

Results

Bacterial cultivation results

After incubation, the minimum value of the total microbial count was 800.00 ± 346.41 CFU/ml, the maximum value

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