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Microflora Profiling of Infected Root Canal before and after Treatment Using Culture-Independent Methods

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This study aimed to profile the microflora in infected root canals before and after root canal treatment using culture-independent methods. Six infected root canals in single-rooted teeth with periapical lesions from five subjects were included. Quantification of total bacteria was performed by real-time PCR with primers targeting 16S rRNA genes. PCR products with universal 16S rRNA gene primers were cloned and partially sequenced, and bacterial identification at the species level was performed by comparative analysis with the GenBank database. The concentration of extracted DNA before treatment was higher than that after root canal treatment, although the difference was not statistically significant. Sequence analysis revealed that oral bacteria such as *Fusobacterium*, *Streptococcus*, *Olsenella*, and *Pseudoramibacter* detected in cases before root canal treatment disappeared after treatment. These results suggest that the root canal microflora are distinct before and after root canal treatment, and that treatment changes the microflora in both quantity and quality.

Keywords: 16S ribosomal RNA, microflora, PCR, root canals

Introduction

Through various anaerobic culturing techniques, it has been shown that the microflora associated with endodontic lesions of human teeth consist of obligate anaerobes, thus suggesting that the environment of the lesions is anaerobic and favors the growth of obligate anaerobes (Sundqvist, 1976; Ando and Hoshino, 1990; Sato *et al.*, 1993). Furthermore, molecular biological methods such as 16S rRNA sequence analysis have shown that the microflora in infected root canals is composed of various microorganisms, including obligate anaerobes (Siqueira and Rôças, 2009). Importantly, it has been reported that *Solobacterium* oral clone, *Bacteroides*-like oral clone and *Pseudoramibacter alactolyticus* are predominant before treatment, while *Streptococcus* species are predominant after treatment with a calcium hydroxide paste (Sakamoto *et al.*, 2007). However, detailed microflora profiles have not been clarified, and the influence of intracanal medicaments other than calcium hydroxide are unknown.

Therefore, in the present study, 16S rRNA sequence analysis was performed in order to profile the root canal microflora qualitatively before and after root canal treatment, using formalin guaiacol as an intracanal medicament. Quantification of total bacteria in root canals was also performed by real-time PCR.

Materials and Methods

Subjects

Subjects with apical periodontitis (two females and three males; age, 25–72 y, mean, 53.0±19.3 y) attending the Clinical Division of Endodontology of Tohoku University Hospital, Sendai, Japan, were randomly selected for this study (Table 1). Apical periodontitis was diagnosed based on clinical features, i.e., putrefactive smell, spontaneous pain, percussion pain, tenderness, pus discharge, swellings and fistula, and radiographic findings (Tronstad, 2009). Two samples (1A and 1B) were diagnosed as apical periodontitis with fistula, while the other four cases were diagnosed as chronic apical periodontitis. Selected teeth had sufficient coronal structure for adequate isolation with a rubber dam, and were free of periodontal pockets deeper than 4 mm. Based on history, all subjects were medically healthy, and received no antibiotics within the 3 months before sampling. Informed consent was obtained from all subjects, and this study was approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry, Sendai, Japan.

Sampling and DNA extraction

At the first visit, each tooth was isolated with a rubber dam, and the surgical field was disinfected with both iodine glycerin dental disinfectants Showa (Showa Yakuhin Kako Co., Ltd., Japan) and 70% ethanol (Tronstad, 2009). A coronal access cavity was prepared with a sterilized high-speed bur under irrigation with sterile saline solution. When the pulp chamber was exposed, a sterile #15 K-file (GC, Japan) was introduced and the canal length was determined using an apex locator (Root ZX, Japan). Dentin samples were collected from an apical canal by intensive

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Table 1. Clinical features of subjects

Subjects	1		2	3	4	5
Samples	1-A	1-B	2	3	4	5
Age	72		42	25	60	66
Gender	Male		Female	Male	Male	Female
Sampling site[a]	21	22	31	22	22	41
Size of lesion[b]	8	2	4	7	4	4

[a] Tooth sampling sites are expressed using the FDI two-digit notation.
[b] Size (mm) of periapical periodontitis lesion estimated by x-ray examination.

filing with a sterile K-file suitable for the canal size.

After the first sampling, cleaning and shaping of the root canal was carried out with sterile K-files (#15 to #55; GC, Tokyo, Japan) under alternative irrigation with 3% H_2O_2 and Antiformin JP Dental (Nihon Shika Yakuhin Co., Ltd., Japan). An intracanal medicament such as Formalin Guaiacol Neo (Neo Dental Chemical Products Co., Ltd., Japan) was applied (Yamauchi *et al*., 2010) until the next appointment, i.e., for 7 to 10 days. The coronal access cavity was sealed with temporary cement (Caviton; GC, Japan).

On the day of root canal obturation, each tooth was evaluated for clinical condition and it was confirmed that there were no clinical signs of apical periodontitis as described above. The tooth was isolated with a rubber dam, and the operative field was disinfected as described above. The temporary cement was removed and the intracanal medicament was rinsed out of the canal with sterile saline solution and a K-file. Immediately prior to root canal obturation with gutta-percha and sealer, a dentin sample was again collected. After obturation, the tooth was temporarily filled with glass ionomer cement (Fuji IX; GC, Japan).

Each file was cut with a sterilized wire cutter and was placed in 300 µl of 150 mM sterile phosphate buffered saline (pH 7.2) in a sterile tightly screw-capped tube at 4°C until use. After vortexing, dentin samples (ca. 0.1 mg) were harvested by centrifugation at 10,000×*g* at 4°C for 10 min and the supernatants were removed. Genomic DNA in the dentin of each sample was extracted using the GeneClean kit for Ancient DNA (Bio 101 Inc., USA) according to the manufacturer's instructions.

Quantification of total bacteria

Quantitative real-time PCR amplification was performed with universal primers for 16S rRNA genes, 357F and 907R (Lane, 1991;Yamaura *et al*., 2005; Abiko *et al*., 2010), and iQ SYBR Green Supermix (Bio-Rad Laboratories, USA), according to the manufacturer's instructions. Primer sequences were: 357F (5′-CTC CTA CGG GAG GCA GCA G-3′) and 907R (5′-CCG TCA ATT CMT TTR AGT TT-3′). Real-time PCR amplification was performed in an iCycler (Bio-Rad Laboratories) programed for 3 min at 95°C for initial heat activation, followed by 40 cycles of 15 sec at 95°C for denaturation, 30 sec at 55°C for primer annealing and 30 sec at 72°C for extension. During the extension step, fluorescence emissions were monitored, and data were analyzed using iCycler iQ software (Bio-Rad Laboratories). Genomic DNA from *Enterococcus faecalis* JCM8728 was used as a standard for quantitative analysis.

16S rRNA gene sequence analysis

For cloning analysis, 16S rRNA genes were amplified by PCR with the primers 357F and 907R, as described above, and *Taq* DNA polymerase (Hot Start *Taq* Master Mix; Qiagen GmbH, Germany) according to the manufacturer's instructions. PCR amplification was performed in an iCycler (Bio-Rad Laboratories) programed for 15 min at 95°C for initial heat activation, followed by 30 cycles of 1 min at 94°C for denaturation, 1.5 min at 55°C for primer annealing, 1.5 min at 72°C for extension and 10 min at 72°C for final extension. PCR products were separated on 1% agarose gels (Certified Low Range Ultra Agarose; Bio-Rad Laboratories) in Tris-borate EDTA buffer (100 mM Tris, 90 mM Borate, 1 mM EDTA, pH 8.4), stained with ethidium bromide and photographed under UV light. PCR product size (ca. 550 bp) was confirmed by comparison with molecular size markers (100-bp DNA ladder; Invitrogen Corp., USA).

PCR products were purified using the GFX PCR DNA and a Gel Band Purification Kit (GE Healthcare Bio-Science Corp., USA), and were transformed into Qiagen EZ Competent Cells using the Qiagen PCR Cloning plus Kit (QIAGEN GmbH). Transformed competent cells were cultured on Luria-Bertani (LB) agar containing 40 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), 0.1 mM IPTG (isopropyl-1-thio-β-D-galactoside) and 100 µg/ml ampicillin. After incubation for 16 h at 37°C, all of the white colonies obtained were transferred to LB liquid medium containing 100 µg/ml ampicillin. After overnight incubation at 37°C, plasmid DNA was purified using the Wizard Plus SV Minipreps DNA Purification System (Promega, USA).

Plasmid DNA was sequenced (at least 530 bp) at Hokkaido System Science Co., Ltd. (Japan) using the T7 promoter primer, the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems Japan Ltd., Japan) and an automated DNA sequencer (PRISM-3100; Applied Biosystems Japan Ltd.). Bacterial identification was performed using BLAST searches of the GenBank in order to identify the closest match to each of the obtained partial 16S rRNA gene sequences. Bacterial species were determined by percent sequence similarity (>98%). Rarefaction curves were generated using Analytic Rarefaction v1.3 (http://www.uga.edu/strata/software/index.html).

Statistical analysis

Wilcoxon test was used to determine the statistical significance of the concentration of extracted DNA determined by quantitative real-time PCR. A p value of <0.05 was considered to be statistically significant.

Table 2. **Number of clones based on 16S rRNA gene sequence analysis obtained from infected root canals at first root canal treatment and prior to root canal obturation**

	GenBank Accession Numbers	Samples	Before treatment (At first root canal treatment)								After treatment (Prior to root canal obturation)							
			1A	1B	2	3	4	5	Total		1A	1B	2	3	4	5	Total	
		Total	11	15	0	12	2	18	58	(100)[a]	27	25	31	6	4	3	96	(100)
Fusobacterium nucleatum	JN052091		2	1		6			9	(15.5)							0	
Streptococcus mitis / oralis	JN052092							4	4	(6.9)							0	
Pseudoramibacter alactolyticus	JN052093			1				3	4	(6.9)							0	
Olsenella profusa	JN052094			3				1	4	(6.9)							0	
Methanobrevibacter oralis	JN052095							3	3	(5.2)							0	
Odoribacter denticanis	JN052096		2	1					3	(5.2)							0	
Veillonella parvula	JN052097							2	2	(3.4)							0	
Bacteroides sp. 22C-like	JN052098		2						2	(3.4)							0	
Uncultured bacterium clone GOR_aag74h11-like	JN052099		2						2	(3.4)							0	
Propionibacterium acidifaciens	JN052100			1				1	2	(3.4)							0	
Deferribacteres sp. oral clone JV006	JN052101			2					2	(3.4)							0	
Nevskia soli	JN052102					1			1	(1.7)							0	
Sphingomonas echinoides	JN052103						1		1	(1.7)							0	
Actinomyces georgiae	JN052104					1			1	(1.7)							0	
Streptococcus sobrinus	JN052105							1	1	(1.7)							0	
Uncultured *Olsenella* sp. clone 1+7-15	JN052106							1	1	(1.7)							0	
Prevotella intermedia	JN052107			1					1	(1.7)							0	
Porphyromonas endodontalis	JN052108			1					1	(1.7)							0	
Treponema socranskii	JN052109		1						1	(1.7)							0	
Rothia aeria	JN052110							1	1	(1.7)							0	
Acholeplasma morum-like	JN052111		1						1	(1.7)							0	
Eubacterium minutum	JN052112		1						1	(1.7)							0	
Pyramidobacter piscolens	JN052113			1					1	(1.7)							0	
Peptostreptococcus stomatis	JN052114			1					1	(1.7)							0	
Adlercreutzia equolifaciens-like	JN052115			1					1	(1.7)							0	
Pseudomonas putida	JN052116						1		1	(1.7)	7	8	8			1	24	(25.0)
Bradyrhizobium japonicum	JN052117					3			3	(5.2)	9	6	7		1		23	(24.0)
Methylobacterium mesophilicum	JN052118								0		8	4	3	1	1		17	(17.7)
Rhodococcus erythropolis	JN052119							1	1	(1.7)		3	2	1	1		7	(7.3)
Uncultured bacterium clone nbw572e08c1	JN052120					1			1	(1.7)	2		2			1	5	(5.2)
Sphingomonas echinoides-like	JN052121								0			1	2	1			4	(4.2)
Pseudomonas fluorescens / reactans	JN052122								0			1	1				2	(2.1)
Bacteroides-like sp. oral clone X083	JN052123			1					1	(1.7)						1	1	(1.0)
Caulobacter crescentus / vibrioides / segnis	JN052124								0					1			1	(1.0)
Uncultured bacterium clone mek64b12	JN052125								0				1				1	(1.0)
Sphingobacterium spiritivorum-like	JN052126								0				1				1	(1.0)
Propionibacterium acnes	JN052127								0					1			1	(1.0)
Methylobacterium dichloromethanicum / extorquens / thiocyanatum	JN052128								0			1					1	(1.0)
Mesorhizobium loti	JN052129								0		1						1	(1.0)
Uncultured bacterium clone nbw643g04c1	JN052130								0			1					1	(1.0)
Aquabacterium hongkongensis	JN052131								0				1				1	(1.0)
Uncultured *Acidovorax* sp. clone A19	JN052132								0				1				1	(1.0)
Massilia brevitalea	JN052133								0				1				1	(1.0)
Herbaspirillum putei	JN052134								0						1		1	(1.0)
Uncultured bacterium clone: TSCOR001_M22	JN052135								0				1				1	(1.0)
Uncultured bacterium clone G-30	JN052136								0					1			1	(1.0)

[a] Percentages are given in parentheses.

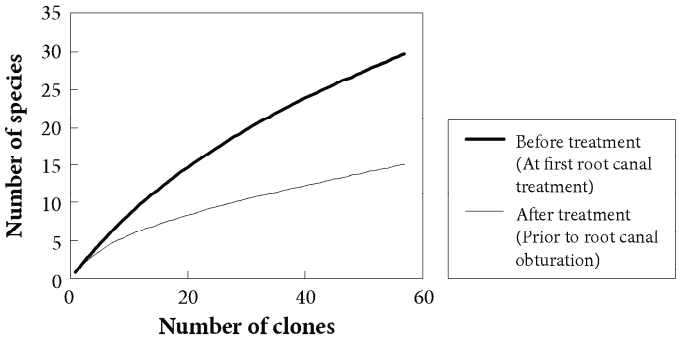

Fig. 1. **Rarefaction curve analysis based on 16S rRNA gene sequence obtained from infected root canals at first root canal treatment and prior to root canal obturation.**

Results

Bacterial DNA was detected in all samples, and thus bacteria were found to be present in all samples both before and after treatment. The concentration of extracted DNA before treatment (mean, 52.7 ng/ml; range, 0.36–152 ng/ml) was higher than that after treatment (mean, 10.9 ng/ml; range, 0.02–36 ng/ml), although the difference was not statistically significant ($p>0.05$).

A total of 58 and 96 clones were obtained from samples before and after treatment, respectively (Table 2). Among the 58 clones before treatment, *Fusobacterium nucleatum* (9 clones, 15.5%) was predominantly detected, followed by *Streptococcus mitis/oralis*, *P. alactolyticus*, and *Olsenella profusa* (Table 2). On the other hand, *Pseudomonas putida*, *Bradyrhizobium japonicum*, *Methylobacterium mesophilicum*, and *Rhodococcus erythropolis* were predominant among the 96 clones after treatment. Based on the rarefaction curves, the bacterial community of samples before treatment was more diverse than that after treatment (Fig. 1).

Discussion

Fusobacterium, as well as *Streptococcus*, *Olsenella*, and *Pseudoramibacter*, were predominant (39.6% in total) before root canal treatment in the present study (Tables 2 and 3), in accordance with previous studies using bacterial culturing (Le Goff *et al.*, 1997; Lana *et al.*, 2001; Vianna *et al.*, 2007) and molecular methods (Jacinto *et al.*, 2007; Blome *et al.*, 2008; Rôças and Siqueira, 2008). These four predominant genera (*Fusobacterium*, *Streptococcus*, *Olsenella*, and *Pseudoramibacter*) appeared to belong to oral bacteria, thus suggesting that these bacteria in root canals are derived from other oral sites, and that these bacteria are among the most common bacteria in root canals, being associated with periapical lesions. *Pseudoramibacter* species were reported to be predominant in a previous study based on 16S rRNA gene sequencing of clone libraries (Sakamoto *et al.*, 2007). In addition, *Pyramidobacter piscolens* and *Peptostreptococcus stomatis*, recently established bacterial species (Downes and Wade, 2006; Downes *et al.*, 2009), and *Methanobrevibacter oralis*, belonging to *Archaea* (Vianna *et al.*, 2006; Vickerman *et al.*, 2007), were detected in cases before root canal treatment in the present study (Table 2). These results were in accordance with previous studies (Downes and Wade, 2006; Vianna *et al.*, 2006; Vickerman *et al.*, 2007; Downes *et al.*, 2009) suggesting an association between microorganisms and endodontic infections.

Pseudomonas, *Bradyrhizobium*, *Methylobacterium*, and *Rhodococcus* were predominant (77.2% in total) after root canal treatment in the present study (Tables 2 and 3), although the findings of the present study did not agree with a previous study reporting the predominance of *Streptococcus* species (Sakamoto *et al.*, 2007). This difference may be due to differences in the intracanal medicaments used. The isolation of *Pseudomonas* species from root canals (0.9–22%) has been reported previously (Nord *et al.*, 1972; Molander *et al.*, 1998; Cheung and Ho, 2001). *Pseudomonas*, *Bradyrhizobium,* and *Rhodococcus* species are known to be able to grow in nutrient-poor conditions such as in soils and water systems (Crist *et al.*, 1984; Ozawa and Doi, 1996; Bell *et al.*, 1998; Saito *et al.*, 1998; Minamisawa and Mitsui, 2000). This may be one of the reasons why these microorganisms survive in root canals even after root canal treatment. *Methylobacterium*, utilizing methanol and methylamine as substrates, has been detected in oral cavities (Anesti *et al.*, 2005), but this bacterium, as well as *Pseudomonas*, *Bradyrhizobium*, and *Rhodococcus*, is not reportedly related to oral diseases such as periapical periodontitis. These bacteria were detected in very small numbers before treatment (8.6% in total) in the present study, thus suggesting that they are minor components of the microflora in root canals and that they survived the treatment.

Table 3. **Predominant bacterial genera[a] based on 16S rRNA gene sequence analysis of infected root canal microflora at first root canal treatment and prior to root canal obturation**

	Before treatment (At first root canal treatment)		After treatment (Prior to root canal obturation)	
Fusobacterium	9	(15.5)[b]	0	(0)
Streptococcus	5	(8.6)	0	(0)
Olsenella	5	(8.6)	0	(0)
Pseudoramibacter	4	(6.9)	0	(0)
Pseudomonas	1	(1.7)	26	(27.1)
Bradyrhizobium	3	(5.2)	23	(24.0)
Methylobacterium	0	(0)	18	(18.8)
Rhodococcus	1	(1.7)	7	(7.3)

[a] Bacterial genera >5% of root canal microflora are listed.
[b] Percentages are given in parentheses.

The bacteria comprising root canal microflora were clearly different before and after root canal treatment (Table 2). More specifically, oral bacteria such as *Fusobacterium*, *Streptococcus*, *Olsenella*, and *Pseudoramibacter* detected in cases before root canal treatment completely disappeared after treatment, while *Pseudomonas*, *Bradyrhizobium*, *Methylobacterium*, and *Rhodococcus* were predominant after treatment (Table 3). In addition, the bacterial diversity of root canal microflora was different before and after root canal treatment (Fig. 1). These differences could be due to the drastic environmental changes in root canals brought about by both mechanical cleaning with dental files and topical application of dental medicaments such as formalin guaiacol.

The present study suggests that root canal treatment alters root canal microflora profiles in both quantity (bacterial amount) and quality (bacterial composition).

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