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Analysis of microflora in dentinal tubule by denaturing gradient gel electrophoresis

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Abstract

This study was performed to apply denaturing gradient gel electrophoresis to microbiological examination of endodontic infections. The method was used to assess the bacterial communities in dentinal tubules. Samples were collected using #15, #35, and #60 K-type files from five infected root canals during treatment. Deoxyribonucleic acid(DNA) was extracted from the samples, and 16S ribosomal DNA was amplified by PCR using universal primers. The polymerase chain reaction(PCR) products were separated in the denaturing gel and band patterns were compared between the deep (#60 file) and superficial layers (#15 file) of the dentin. The major bands were then excised and DNA fragments in the gel were cloned and sequenced. The sequence data were subjected to BLAST search in the GenBank database for determination of bacterial species or closest relatives. In three root canals, similar band patterns were observed in both superficial and deep layers of the dentin, while several specific bands remained in the deep layer in two roots. The bacteria isolated from the deep layers were *Porphyromonas gingivalis*, *Olsenella profuse*, *Atopobium rimae*, and *Prevotella*, *Flexistipes*, Firmicutes, *Peptostreptococcus*, *Dialister*, and *Eubacterium* spp. Unlike previous studies, gram-negative anaerobic rods were isolated from the deep layers. Clone library analysis was simultaneously performed and similar results were obtained. The method utilized here will be useful for microbiological examination of endodontic infections. In addition, although it is still unknown whether they were viable, this study demonstrated the presence of gram-negative rods in dentinal tubules.

Keywords: Denaturing gradient gel electrophoresis (DGGE); 16S ribosomal ribonucleic acid(RNA); Dentinal tubule; Endodontic infection

1. Introduction

Advances in molecular biological techniques have enabled the detection of uncultivable bacteria in root canals and revealed that bacterial community structure is more diverse than previously thought based on culture methods [1, 2, 3]. The dentinal tubule microflora has been characterized using culture-based techniques [4, 5, 6]. Although wide ranges of bacteria, including gram-negative anaerobes, were found in root canals, relatively limited bacterial species were isolated from the deep dentinal tubules. Gram-positive obligate anaerobes, mainly strains belonging to the genera, *Lactobacillus* and *Streptococcus*, have been reported to be predominant in the deeper dentinal tubule. These bacteria were isolated from areas 0.5–2.0 mm from the canal-dentin boundary [7]. Gram-negative anaerobes, such as *Porphyromonas*, *Fusobacterium*, and *Prevotella* species, have been suggested to be associated with acute symptoms of apical lesions. However, they have not been isolated from the deep dentinal tubule [7]. Therefore, more sensitive analysis is required to determine the true diversity of microflora in the dentinal tubule, especially deeper within the tubule.

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PCR is now widely used for microbiological diagnosis in various infectious diseases. However, the method still has some limitations. Even for polymicrobial communities, such as those in the root canal, PCR targets have to be selected, and therefore the bacterial diversity of the whole community remains unclear. A new approach for polymicrobial ecology is based on 16S ribosomal DNA (16S rDNA) information obtained by clone library analysis [2, 3]. The method appears to be very useful for determining the composition of bacterial communities. However, the analysis requires a number of laborious cloning and sequencing tasks.

Denaturing gradient gel electrophoresis (DGGE) was originally described for mutation analysis [8, 9]. Recently, this molecular technique has been shown to be useful in microbial ecology [10]. As sequence-specific separation of 16S rDNA and further sequencing or hybridization analysis are possible, DGGE has become a powerful tool to examine bacterial diversity in various natural habitats, such as marine or soil environments [11]. This method has also been employed in the medical and dental fields for analysis of polymicrobial communities in humans [12, 13, 14].

Bacteria in the root canal play essential roles in apical periodontitis, and the remaining bacteria in dentinal tubules may be associated with persistent endodontic infections [16]. Understanding the microflora in the root canal and dentinal tubule is indispensable for the improvement of treatment strategies for endodontic infection. In the present study, the DGGE method was applied to microbiological examination of endodontic infections. The profiles of the bacterial communities in both root canals and dentinal tubules were simultaneously analyzed using DGGE.

2. Material and methods

2.1. Collection of endodontic samples

Samples were collected from five teeth from five patients. Single-rooted teeth with periapical radiolucency (2.0 – 3.0 mm in diameter) and without severe periodontal disease (periodontal pockets < 4 mm) were selected for the study. None of the root canals had been treated previously with endodontic therapy. They had carious lesions and necrotic pulp. Four teeth were asymptomatic except for slightly tenderness to percussion. Dental fistula was observed in the remaining one case. The diameter of all canals near the root apex was almost the same as that of a #15 K-type file (Dentsply/Maillefer).

The selected teeth were isolated with a rubber dam, carious lesions were removed, and cleansed with 3% hydrogen peroxide. Complete access preparations were made, and the third of the root canal from the orifice was enlarged into a funnel-shape using a Pesso reamer (Pierce). Samples were collected initially with a #15 K-type file. The file was introduced to a level approximately 0.5 mm short of the tooth apex by monitoring with an electric apex locator (Morita), and the necrotic pulp and dentin powder attached to the file were suspended in 1 ml of PBS (−) (Gibco-BRL). Canal preparation was performed using K-type files and the canal was irrigated with 3% hydrogen peroxide and 2.5% sodium hypochlorite between each file size. Second and third samples were collected by #35 and #60 K-type files in the same way. The Ethics Committee of Osaka Dental University approved the protocol of this study (approval number: 110972), and all patients provided written informed consent to participate in the study.

2.2. DNA extraction

Total bacterial DNAs were extracted from the clinical samples using InstaGene Matrix (Bio-Rad) according to the manufacturer's instructions. Briefly, bacterial samples in 1 ml of PBS (−) (Gibco-BRL) were pelleted and resuspended in 100 µl of InstaGene Matrix (Bio-Rad). The suspension was incubated at 56°C for 30 min and then 100°C for 8 min. After incubation, the suspension was centrifuged and 5 µl of the resulting supernatant was used for PCR prior to DGGE.

2.3. PCR-DGGE analysis

The 16S rDNA fragments (nucleotide positions 341 – 926 in the *Escherichia coli* sequence) were amplified with two conserved primers. A forward primer with a 40-bp GC clamp added to its 5′-end (5′-cgcccgccgcgccccgcgcccgtcccgccgccccccgcccg-cctacgggaggcagcag-3′) and reverse primer (5′-ccgtcaattcctttRagttt-3′) were prepared for PCR. Amplification was carried out using a High-Fidelity PCR Master Kit (Roche Molecular Biochemicals) in 50-µl reactions. A touchdown procedure (65°C – 55°C annealing temperature) was adopted for thermocycling [17].

The DCode Universal Mutation Detection System (Bio-Rad) was used for gel electrophoresis. The PCR products (20 µl) were loaded onto an 8% (w/v) polyacrylamide gel containing a linear gradient ranging from 20% to 50% denaturant (7 M urea and 40% formamide). The gel was run for 3 hours at 200 V in 1× TAE buffer (0.04 M Tris-acetate/1 mM EDTA, pH 8.5) at a constant temperature of 60°C. After electrophoresis, the gel was stained with ethidium bromide, and the amplified DNA bands were visualized with a Fluor-S MultiImager (Bio-Rad).

2.4. Sequence analysis of DGGE bands

The major bands on the denaturing gel were excised, purified, and cloned into the PCR 2.1 vector (Invitrogen). The insert DNA was sequenced using an ABI Prism BigDye terminator cycle sequencing ready reaction kit (Perkin Elmer) and an automated DNA sequencer (ABI PRISM 377; Perkin Elmer). The sequence data were subjected to BLAST search in the GenBank database.

2.5. Clone library analysis

Microflora in one of the five root canals was simultaneously evaluated by clone library analysis. For construction of the library, 16S rDNA was amplified using the universal primers 27F and 1525R. The amplified 16S rDNA fragments were ligated into the PCR 2.1 vector, and *E. coli* XL1-blue was transformed with the ligation mixtures. The transformants were randomly picked up and plasmids were isolated. Sequencing and BLAST search were performed as described above.

3. Results

Prior to DGGE analysis, PCR products were subjected to electrophoresis in 1.5% agarose gels. A single band was detected from each sample at the expected size (585 bp, data not shown). The DGGE profiles of the amplified 16S rDNA are shown in Figure 1.
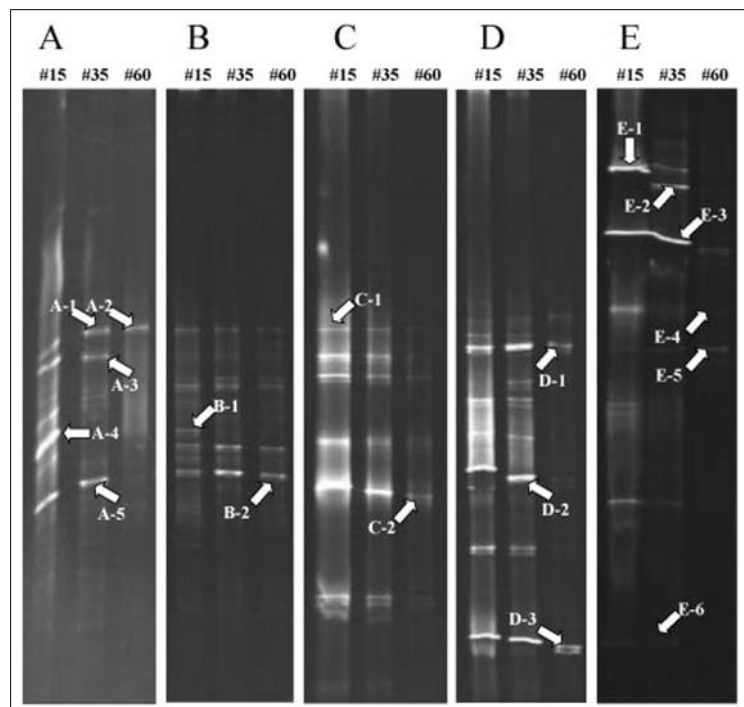

Figure 1 DGGE profiles of amplified 16S rDNA

Samples were collected from five root canals (A – E) at different distances from the canal-dentin boundary during root canal treatment with #15, #35, and #60 K-type files, and were subjected to DGGE analysis as described in the Materials and methods. Eighteen intense bands indicated by arrows were cloned and sequenced. The bacterial species identified from the sequence data are shown in Table 1.

The multiple 16S rDNA fragments amplified by PCR were separated according to sequence specificity. Distinct band patterns were observed among the different root canals, although several bands were common to more than two canals (A-1 and C-1; A-3 and D-1; A-5, B-2 and D-2). From the surface to the deep dentin layer, the DGGE profile did not change dramatically in root canals B, C, and E. However, in root canals A and D, several selected bands remained in deep dentin layer (bands A-2, D-1, and D-3).

To determine the dominant species in each sample, selected major bands were cloned and sequenced. The selected bands are indicated by arrows in Figure 1 (numbered A – G). Four clones were isolated and sequenced from each band and the nucleotide sequences (250 – 300 bp) were compared with 16S rDNA in the database. All sequence data of the

clones were closely affiliated with existing sequence data in GenBank with similarity values of 98% – 100%. The identified bacterial species are shown in Table 1.

Table 1 Identified bacteria from DGGE bands

Sample (DGGE bands[a])	Phylotype/species	Accession no.	No. of isolated clones (Four clones from each band)
A-1	*Porphyromonas gingivalis*	AF414809	4
A-2	*Porphyromonas gingivalis*	AF414809	4
A-3	Firmicutes sp. oral clone	AF287778	4
A-4	*Lautropia* sp. oral clone	AY134902	4
A-5	*Flexistipes*-like sp. oral clone	AY005444	4
B-1	*Fusobacterium* sp. oral clone	AF287805	1
	Fusobacterium nucleatum	AF543300	3
B-2	*Flexistipes*-like sp. oral clone	AY005444	4
C-1	*Porphyromonas gingivalis*	AF414809	4
C-2	Uncultured fiber-attaching rumen bacterium	AB045742	4
D-1	Firmicutes sp. oral clone	AF287778	4
D-2	*Flexistipes*-like sp. oral clone	AY005444	4
D-3	*Olsenella profuse*	AF292374	4
E-1	*Lachnospiraceae* oral clone	AF481220	3
	Staphylococcus sp. B3117	AF322002	1
E-2	*Alpha Proteobacterium* sj22	AF530921	2
	Propionibacterineae bacterium clone RH-B24N	AF513962	2
E-3	*Peptostreptococcus* sp. oral clone	AF287763	2
	Dialister sp. oral isolate	AF481209	1
	Atopobium rimae	AF292371	1
E-4	*Prevotella buccae*	L16477	2
	Prevotella nigrescens	AF414844	2
E-5	*Eubacterium* sp. oral clone	AF287764	4
E-6	*Olsenella profuse*	AF292374	4

[a] Corresponding to the bands in Figure 1.

Porphyromonas gingivalis, *Olsenella profuse*, *Atopobium rimae*, and *Prevotella*, *Flexistipes*, Firmicutes, *Peptostreptococcus*, *Dialister*, and *Eubacterium* spp. were identified in the deep layers. Among them, *P. gingivalis*, Firmicutes, *Flexistipes*, and *Olsenella* spp. were identified from multiple root canals. Band A-1 indicated that *P. gingivalis* was still dominant in the deep layer (A-2), while other dominant bands in sample A disappeared during endodontic therapy. Similarly, D-1 indicating Firmicutes sp. and D-3 indicating *Olsenella* were selectively dominant in the deep dentin layer.

Clone library analysis was performed using the samples from root canal E. Thirty clones were isolated and sequenced in each layer, and were identified to the genus level by comparison with the DNA database. The results are shown in Table 2.

Table 2 Isolated bacteria by clone library analysis (sample E)

Bacterial species	[a]Number of clones		
	#15	#35	#60
Peptostreptococcus sp.	8	10	8
Dialister sp.	6	10	7
Lachnospiraceae sp.	4	4	1
Firmicutes sp.	3	4	1
Atopobium sp.	2	2	3
Olsenella sp.	2	2	2
Methylobacterium sp.	1	1	4
Eubacterium sp.	2	2	2
Propionibacterium sp.	1	0	1
Unknown	1	2	1

[a] the total number of isolated clones was 30 in each sample of #15, #35, and #60

Clones with sequences identity < 98% were found in some cases, and are shown as "unknown." The bacterial community structures of different dentin layers were almost the same and were dominated by *Dialister* and *Peptostreptococcus* spp. The dominant bacteria isolated by clone library analysis and DGGE method were almost identical.

4. Discussion

As wide ranges of bacterial species have been isolated from root canals [3, 4, 18, 19], DGGE showed multiple bands, and the band patterns were different between the sampled teeth (Fig. 1). The results indicated the presence of complex polymicrobial communities in root canals, and that the composition of microorganisms was different in each root canal or patient. On the other hand, several major bands were common among different samples. The relative quantity of DNA in the individual bands generally suggested numerical dominance. Bacterial species dominating the multiple root canals, such as *P. gingivalis*, Firmicutes, and *Flexistipes*, may be adapted to the environment of the root canal. Consistent with previous reports [3, 18, 19], the Firmicutes phylum (Table 1) was dominant in most cases. In contrast, the microflorae of the deep dentin layers were inconsistent with previous reports. Using culture-based techniques, the microflorae of the deep dentin layers were reported to be dominated by gram-positive anaerobes, such as lactobacilli and streptococci [7]. It has also been suggested that gram-negative anaerobic rods are not able to invade dentinal tubules [20]. However, in this study, gram-negative rods, such as *Porphyromonas* and *Prevotella*, were found to be dominant in the deep layers of the dentin. *Dialister*, which is a current topic of interest in periodontal disease [21, 22, 23, 24], was also identified in the deep layer. Supporting our results, these bacteria have been suggested to have the ability to invade the dentinal tubule [25], as they can penetrate into the dentin wall from the periodontal ligament side [26]. The gram-negative anaerobes are thought to co-invade with streptococci or other facultative anaerobes [26]. It should also be noted that only several major bands in each sample were sequenced in this study and many unidentified bands remained in the deep dentin layer, which may include lactobacilli or streptococci. The bacterial community structure in dentinal tubules appeared to be more diverse than previously thought based on culture methods, and the present study using DGGE suggested that gram-negative anaerobes can invade into the dentinal tubule from the pulpal side. Limitations of culture-based techniques may have affected the results of previous studies, leading to the underestimation of bacterial diversity.

We compared the DGGE profiles between the superficial (#15 file) and deep dentin layers (#60 file) to investigate whether specific bacteria were selected to penetrate the dentin wall from among the whole bacterial community of the original root canal. In root canal A (Fig. 1), *P. gingivalis* bands (A-1, A-2) were observed throughout the therapy with similar intensity, whereas other bands disappeared in the deep layer. Similarly, Firmicutes (D-1) and *Olsenella* (D-3) were selected in root canal D. In contrast, the band pattern did not change from surface to deep layers in other root canals although bacterial numbers (density of each band) gradually decreased. Interestingly, *P. gingivalis* of root canal A was specifically selected from among the whole bacterial community and invaded into the deep layer, while the population of *P. gingivalis* of root canal C gradually decreased from the surface to the deep layer. Specific bacteria were selected and invaded dentinal tubules in two cases. However, the microflorae in dentinal tubules were similar to those

of the original root canals in the other three cases. We speculated that bacterial cell–cell interactions among the different strains may be important for invasion. Further detailed studies of the microbial community structure in larger samples are required. Sample E from a tooth with fistula demonstrated a distinct band pattern from the others with bands at high molecular weight positions. Investigation of the relation between the clinical aspects and DGGE profile will also be necessary.

Clone library analysis was performed to assess the utility of DGGE. The identified bacteria were mostly common between the two methods. The results demonstrated that DGGE could be a powerful tool for evaluation of polymicrobial community structures in cases of endodontic infection. Clone library analysis is a reliable technique, but is not practical for routine examinations as it requires numerous sequencing steps. Therefore, DGGE may have a methodological advantage for clinical examinations.

The present study demonstrated the great potential of DGGE for determining the polymicrobial community structure in cases of endodontic infection. However, several minor limitations should also be discussed. For example, the 16S rDNA band in DGGE sometimes reflects the presence of multiple microbial species [10]. In fact, several species were identified from single bands in the DGGE profile of sample E. It may be difficult to separate the 16S rDNA bands completely when closely related species are present in large numbers. To resolve these problems, primer design or electrophoresis conditions should be optimized. Another problem is related to the viability of identified bacteria. As genomic DNA could be recovered from non-viable microorganisms, the method used in this study was not able to assess the viability of the isolates. Nevertheless, the presence of DNA in the deeper tubule would suggest active invasion of these bacteria in the clinical course of endodontic infections.

DGGE was able to express the complicated microflora visually with its band patterns, and sequencing of the major bands revealed the dominant bacteria in the dentinal tubules. The presence of gram-negative anaerobic rods in the dentinal tubule may require more attention for disinfection during endodontic therapy.

5. Conclusion

The DGGE method and clone library analysis were applied for microbiological examination of infected root canals, and both methods revealed the presence of gram-negative anaerobic rods in the deep dentin layers. The DGGE method has potential to become a powerful tool for analyzing the changes in the microflora in root canals through endodontic therapy.

Compliance with ethical standards

Disclosure of conflict of interest

The authors have no conflicts of interest to clear.

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