Abstract: We used denaturing gradient gel electrophoresis (DGGE) to compare bacterial profiles in periodontium and root canals of teeth with combined periodontal-endodontic lesions. Samples of dental plaque and necrotic pulp were collected from thirteen extracted teeth with advanced periodontitis. Genomic DNA was extracted for polymerase chain reaction (PCR) analysis using universal bacterial primers. The PCR products were then loaded onto DGGE gels to obtain fractionated bands. Characteristic DGGE bands were excised and DNA was cloned and sequenced. The number of bands, which indicates the number of bacterial species, was compared between dental plaques and necrotic pulp tissues from the same tooth. Although the difference was statistically significant ($P < 0.01$), there was no positive correlation; similarity (Dice coefficient) was 13.1% to 62.5%. Some bacteria species were present in both the periodontal pockets and root canals of the same tooth; however, periodontal bacteria did not always invade the root canals, and some bacteria in root canals were not present in periodontal pockets of the same tooth. In some teeth, unique bacteria in root canals had not passed from periodontal pockets. A basic local alignment search tool (BLAST) sequence search in Genbank indicated that new bacteria species were present in periodontal pockets and root canals. Their characteristics must thus be further analyzed. (J Oral Sci 55, 287-291, 2013)

Keywords: 16S rRNA gene; advanced periodontitis; periodontium; root canal.

Introduction

Periodontitis and pulpal-periapical diseases are caused by mixed infection (mostly by anaerobes) and subsequent inflammation and immune reaction. The pulp and periodontium have embryonic, anatomic, and functional inter-relationships that can lead to worsening of disease, resulting in combined periodontal-endodontic lesions. Using different methods, the lesions can be classified according to etiology, pathologic mechanisms, and clinical manifestation (1-4).

The diseased tooth coexists with periodontal disease and pulpal lesions, the infection from pulpal-periapical diseases, or from periodontal, or both. As part of a simplified procedure, we chose periodontal-resource lesions as our research goal. Teeth extracted due to chronic periodontitis can be used as a lesion model. The adverse effects of periodontal disease on pulp have been debated for many years. Sheykhrezae et al. found that complete necrosis of dental pulp occurred only when the depth of an adjacent periodontal pocket reached the apical third of the root (5). De Deus reported that lateral canals were present predominantly in the apical third (6). These studies suggest that dental pulp in the apical third could be a crucial zone, a border of pulpal change. Therefore, when caries-free teeth with advanced periodontitis and clinical necrosis (as determined by thermal pulp testing) are selected, the periodontal pocket should be deep (>5
mm) and at least one locus of the tooth should be probed to the root apex. In addition, pulpal tissue that appears as brown powder during sample preparation indicates the presence of dead pulp.

To date, most bacterial studies of periodontal and periapical disease have used culture methods; however, such methods are not suitable for detecting uncultivable bacteria or unknown microorganisms (7,8). Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) can overcome the limitations of culture and can be used to evaluate the microbial community. PCR-DGGE allows simultaneous visualization of the profiles of bacterial communities in multiple clinical specimens, even those with unknown bacteria (9-12).

Materials and Methods

Subjects and sample collection

Thirteen teeth were selected from adult patients with advanced periodontitis who were referred to the Department of Periodontics, Shandong University School of Stomatology for periodontal therapy. The teeth were all removed as part of treatment for severe periodontitis. All teeth had deep periodontal pockets (at least one locus probing to the apical zone), necrotic pulp detected on thermal testing, radiographic evidence of periradicular bone loss, and no caries. Our research was approved by the ethical committee of the Shandong University School of Stomatology (2010021), and the study was undertaken with the understanding and written consent of each participant.

Dental plaque samples were taken from the deepest periodontal pocket before tooth extraction. The residue of necrotic dental pulp was collected after tooth extraction, according to the technique of Braga (13) and Chugal (14), and stored in phosphate-buffered saline at −20°C. The 13 patients (9 men, 4 women) were aged 38-64 years (average 49.5 years). They had no systemic diseases and had not used antibiotics for at least 3 months. Smokers were excluded.

DNA extraction

Each clinical sample underwent DNA extraction. Briefly, samples were thawed to 37°C for 10 min and centrifuged for 30 s. The microbial suspension was pelleted by centrifugation at 10,000 rpm for 2 min, and DNA was extracted using a TIANamp Bacteria DNA Kit (Tiangen, Beijing, China). Finally, the total bacterial DNA was eluted with 100 µL of Tris-EDTA buffer (Tiangen). DNA samples were stored at −20°C.

PCR amplification

PCR amplification was performed in a gradient thermocycler (Thermocycler Personal, Whatman Biometra, Göttingen, Germany). For amplification, the following universal bacterial primers were used: 968f (5′-AAC GCG AAG AAC CTT AC-3′) containing a 40-base pair (bp) GC clamp (5′-CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG G-3′) added to its 5′-end, which makes it suitable for DGGE, and 1401r (5′-CGG TGT GTA CAA GAC CC-3′). The sequence of primers has been previously published (12,15). The PCR mixture (50 µL) comprised 10 µL of the DNA extracts from clinical samples, 30 pmol of universal primers, and 25 µL of 2 x EasyTaq PCR SuperMix (TransGene Biotech, Beijing, China). The temperature profile included initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 55°C for 30 s, primer annealing at 56°C for 30 s, extension at 72°C for 35 s, and a final step of 72°C for 10 min.

DGGE

DGGE of PCR products generated with the 968f-GC/1401r primer set was performed using the DCode Universal Mutation Detection System (Bio-Rad, Richmond, VA, USA) at 100 V and 60°C for 3.5 h in 1 x TAE buffer (40 mm Tris-acetate [pH 7.4], 20 mm sodium acetate, 1.0 mm disodium EDTA). The PCR products (35 µL) were loaded on 6% (w/v) polyacrylamide gels containing a linear gradient of the denaturants urea and formamide, increasing from 40% at the top of the gel to 60% at the bottom (100% denaturant corresponded to 7 M urea and 40% [v/v] formamide). Two gels were run to accommodate all samples. After electrophoresis, gels were stained with SYBR green I nucleic acid gel stain (Beyotime, Shanghai, China) for 40 min and then scanned using an ImageLab system (Bio-Rad).

DGGE analysis

DGGE banding patterns were transferred to Quantity One software (Bio-Rad) for construction of a similarity matrix and cluster analysis. Data were statistically analyzed using the paired t-test and Spearman rank correlation analysis in SPSS 17.0. Bands per lane was used as a measurement of microbial diversity, and values for the periodontal pocket and pulpal sample from the same tooth were compared. Separated bands were classified as common or exclusive, ie, present in both lesions or only in samples of necrotic pulp, respectively. A coefficient of similarity (Dice coefficient) was calculated to compare the compositions of two samples. Cluster analysis using the unweighted pair group method with arithmetic aver-
ages (UPGMA) was conducted to determine whether samples had a non-random pattern and to compare DGGE banding patterns.

The Dice coefficient was used to analyze similarity between two lesions (using the Quantity One software) and to determine the composition of bacteria. Analysis of the Dice coefficient was performed as previously described (15).

Excision and sequence analysis of products

Some bands of interest (based on intensity, dynamics in sequential samples) were excised from the gel, put into tubes containing 40 µL sterile milli-Q water, and left at 4°C for 24 h. A volume of 1 µL was used as a template for PCR with the 968f/1401r primer set. The products were checked by agarose gel electrophoresis and then cloned and sequenced using an ABI 3730XL DNA high-throughput DNA sequencer. Taxa were identified by using the Basic Local Alignment Search Tool (BLAST).

Results

DGGE images revealed complicated bacterial profiles in periodontal-endodontic lesions. A large number of bacterial bands were seen in each lane, but only intense bands were analyzed. A representative DGGE gel is displayed in Fig. 1, and Table 1 shows the number of bands from each sample. The mean (SD) number of bands detected in thirteen paired samples was 12.31 (2.93) for the periodontal and 6.92 (2.10) for necrotic pulp tissue (paired t-test, P < 0.01). However, the Spearman rank correlation ratio was 0.109 (P = 0.362). In intragroup comparison (Table 1) 11.8% (2/17) to 55.6% (5/9) of bands in periodontal samples reappeared in pulpal samples. Excepting one sample, exclusive species were present in pulpal samples (16.7% [1/6] to 60% [3/5]).

The similarity (Dice coefficient) of the bacterial profiles for dental plaque and necrotic pulp sampled from the same tooth was 13.1% to 62.5%. A representative DGGE gel is shown in Fig. 1. Cluster analysis of 6 paired samples showed great variability in the bacterial population regardless of sampling position. Two large clusters of interest were evident: one comprised 5 of 6 periodontal samples (with one pulpal “crasher” sample) and the other comprised 5 of 6 samples of necrotic pulp (with one periodontal “crasher” sample) (Fig. 2).

To identify possible strains, some DGGE bands were randomly selected, cloned, and sequenced, as shown in Fig. 1. Seven clones were isolated from each selected band. Taxa identified through BLAST (≥98% identity) consisted of Campylobacter, Fusobacterium, Neis-
seria, Peptostreptococcus, Veillonella, Aggregatibacter, Enterobacter, and Haemophilus in dental plaque and Mogibacterium, Corynebacterium, Neisseria, and Actinomyces in necrotic pulp (Table 2). Approximately half of the BLAST results had an identity less than 95%, including Shuttleworthia, Robinsonella, Actinobaculum, and Bacillus in periodontium and Moryella in necrotic pulp.

**Discussion**

We analyzed the profile of bacteria in samples of dental plaque and necrotic pulp from patients with periodontal-endodontic combined lesions. The similarity of bacteria in dental plaque and necrotic pulp ranged from 13.1% to 62.5%, which indicates overlap in bacterial profiles. Our results show that some periodontitis-related bacteria in deep periodontal pockets can invade the adjacent root canal and cause pulpitis. As compared with necrotic pulp samples, dental plaque samples had more bacterial species ($P < 0.01$), because the periodontium is more exposed to the oral environment. The pulp chamber is a relatively closed environment, and runs through the apical foramen and lateral canals from the oral cavity. All the samples in our research were from patients with ascending pulpitis, without access between root canals and the oral environment. Samples from necrotic pulp also had unique bands indicating that some bacteria may have passed from the periodontium before invading the pulp. Our results also indicate that numerous bacterial species are present in periodontal pockets; however, we did not find evidence of considerable periodontal–endodontic communication of bacterial species in root canals with pulpitis. These observations confirm those of Alves et al. (16).

The species identified in this study differed somewhat from the usual bacterial profile. This discrepancy is due to the following reasons. First, the sample size was small, and only some bands were selected for sequencing and identification. Therefore, recognized pathogenic bacteria may have been missed. Second, molecular methods are able to detect uncultivable bacteria such as Mogibacterium timidum and thus may expand the range of possible pathogenic bacteria. Third, molecular methods have limitations, such as misreading of base sequences. Lastly, the bacterial profiles of some diseases may be related to anatomic features. Because the newly identified bacteria in this study differ from well-known species, additional experiments to determine the virulence of these bacteria are necessary. For example, Actinomyces odontolyticus is an opportunistic pathogen in liver abscesses and hydrothorax. It was detected in samples of necrotic pulp and may be a potential pathogen in combined lesions.

In this study, the DNA of collected samples was cloned, sequenced, and identified using a BLAST search of GenBank on PubMed. Ledder et al. and Zijnge et al. used multiple PCR and DNA hybridization technology (species-specific) to identify DGGE strips (17-18). Other researchers compared electrophoresed bands with known bacteria (19-20). The present method of cloning and sequencing is advantageous because it can detect uncultivable and unknown bacteria. For example, approximately half of the present BLAST results showed less than 95% similarity with well-known bacteria, which indicates that

<table>
<thead>
<tr>
<th>Bands in Fig. 1a</th>
<th>Identified species</th>
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<tbody>
<tr>
<td>A</td>
<td>Aggregatibacter aphrophilus; Peptostreptococcus stomatitis; Veillonella rogosae;</td>
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<tr>
<td></td>
<td>Campylobacter rectus; Neisseria elongata; Veillonella rogosae; Fusobacterium caninellum;</td>
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<tr>
<td>B</td>
<td>Campylobacter concisus; Neisseria elongata; Veillonella rogosae;</td>
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<tr>
<td></td>
<td>Haemophilus parainfluenzae Peptostreptococcus stomatitis;</td>
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<tr>
<td></td>
<td>Enterobacter asburiae; Aggregatibacter aphrophilus;</td>
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<tr>
<td>C</td>
<td>Corynebacterium matruchotii; Neisseria bacilliformis;</td>
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<tr>
<td></td>
<td>Actinomyces odontolyticus;</td>
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<tr>
<td>D</td>
<td>Mogibacterium timidum</td>
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</table>
unknown species were detected using our technique.

In the present samples of combined periodontal-endodontic lesions (of periodontal origin), some bacteria species were present in both the periodontal pocket and root canals; however, not all periodontal bacteria species were present in samples of necrotic pulp. In addition, in necrotic pulp samples, we identified unique bacteria species that completely differed from those in periodontal plaque. Clearly, many new bacteria species remain to be identified and examined for pathogenicity in periodontal pockets and root canals.

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**References**