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Molecular Characterization of the Microbiota Residing at the Apical Portion of Infected Root Canals of Human Teeth

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Abstract

Introduction—This study investigated the bacterial communities residing in the apical portion of human teeth with apical periodontitis in primary and secondary infections using a culture-independent molecular biology approach.

Methods—Root canal samples from the apical root segments of extracted teeth were collected from 18 teeth with necrotic pulp and 8 teeth with previous endodontic treatment. Samples were processed for amplification via polymerase chain reaction (PCR) and separated with denaturing gradient gel electrophoresis (DGGE). Selected bands were excised from the gel and sequenced for identification.

Results—Comparable to previous studies of entire root canals, the apical bacterial communities in primary infections were significantly more diverse than in secondary infections ($p=0.0003$). Inter- and intra-patient comparisons exhibited similar variations in profiles. Different roots of the same teeth with secondary infections displayed low similarity in bacterial composition, while an equivalent sample collected from primary infection contained almost identical populations. Sequencing revealed a high prevalence of fusobacteria, *Actinomyces* sp. and oral *Anaeroglobus geminatus* in both types of infection. Many secondary infections contained *Burkholderiales* or *Pseudomonas* sp. both of which represent opportunistic environmental pathogens.

Conclusion—Certain microorganisms exhibit similar prevalence in primary and secondary infection indicating that they are likely not eradicated during endodontic treatment. The presence of *Burkholderiales* and *Pseudomonas* sp. underscores the problem of environmental contamination. Treatment appears to affect the various root canals of multi-rooted teeth differently, resulting in local changes of the microbiota.

Keywords

Apical periodontitis; endodontic infections; community profiling; polymerase chain reaction; denaturing gradient gel electrophoresis

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The authors deny any conflicts of interest

INTRODUCTION

Apical periodontitis is primarily the result of bacterial infection of the pulp and the root canal system as demonstrated in numerous studies (1–3). To fully comprehend the pathogenesis of apical periodontitis and thus develop more effective strategies for root canal therapy, it is necessary to understand the composition of the microbial community present in the root canal systems of diseased infected teeth. This is especially important since pre-operative apical periodontitis is one major indicator of post-treatment healing or treatment failure (4–7).

In wide range of *in vivo* (human and animal) and *in vitro* (extracted teeth) studies, endodontic samples were collected from various sites, including root canals of clinical patients (4, 6) root canals of experimental animals (2, 8,9), and root canal fragments of extracted human teeth (10–15). In general, the clinical diagnoses selected for studies of human teeth include pulp diagnoses of pulp necrosis and previously treated teeth, and periapical diagnoses that include normal periapex, asymptomatic, symptomatic apical periodontitis and acute apical abscess. Over the past decade the analysis of endodontic microbiota experienced a shift from culture-based to molecular approaches. The growing number of studies employing molecular methods for identification of the microbiota involved in primary as well as secondary endodontic infections augmented the breadth of information on the microbiology of endodontic infections and initiated a shift in the understanding of their etiology (16, 17). In particular, 16S rDNA gene sequencing based techniques were employed to evaluate the members of diverse microbial communities including uncultivated microorganisms. Rocas *et al.* (18) introduced polymerase chain-reaction-based denaturing gradient gel electrophoresis (PCR-DGGE) to investigate the bacterial communities associated with asymptomatic and symptomatic endodontic infections. To date, PCR-DGGE which is a powerful tool for surveying entire bacterial communities in a given ecosystem without cultivation has been applied in several other studies to profile the microbiota associated with primary endodontic infections (10, 13,19–21). Importantly, molecular approaches identified putative endodontic pathogens that were not previously found with culture-based methods.

Differences in microbial diversity between primary and secondary infections and the polymicrobial nature of root canal infections have been demonstrated first by classic culture-based microbiologic methods (3, 4,9,22,23) and were later confirmed by more contemporary molecular biology techniques (16, 17). The microbiota is not homogeneous throughout the length of canal but shifts its composition from coronal to apical portion (8, 10,12,13) which is dominated by obligate anaerobes (11, 21) whose number increases with time (8).

The bacteria in the apical portion are thought to induce damage to the periradicular tissues and cause bone destruction (11, 14,24,25). The apical anatomy of the root canal system and corresponding level of instrumentation/disinfection as well as the level of root filling have been considered important from a treatment point of view (24, 26,27). Despite the strategic importance of the apical portion of the root in endodontic therapy and the known shift in bacterial composition throughout the canal, only relatively few studies have analyzed this region separately (8, 10–14,21). Consequently, we focused in this study on surveying the bacterial communities that reside in the apical portion of infected root canals of human teeth associated with apical periodontitis in primary and secondary endodontic infections. We also evaluated inter- and intra- individual variability, and examined local microbial changes in different roots of multi-rooted teeth.

MATERIALS AND METHODS

Patient Selection and Sample Collection

Teeth were collected from patients scheduled for extraction at the Oral and Maxillofacial Surgery Clinic, UCLA School of Dentistry. The study sample comprised eighteen teeth with necrotic pulp and apical periodontitis (primary infections) and seven previously root canal treated teeth with periradicular lesion (secondary infections). Two non-carious, restoration-free third molars served as controls. Excluded from the study were individuals under the age of 18 years, and those suffering from cancer, diabetes and other immunodeficiency disorders. Also excluded were teeth with marginal periodontal disease. The study protocol was approved by the Office for Protection of Research Subjects at the University of California, Los Angeles, IRB#07-05-054-01.

Samples were collected according to published protocols (11, 14). Immediately following extraction, each tooth was placed into sterile RPMI 1640 tissue culture medium and frozen at -80°C for storage. To collect the root canal microbial samples, teeth were thawed at room temperature (25°C) for 10 min and all attached soft tissue was removed from the roots with a sterile #15 scalpel. The root surface was disinfected with 6% sodium hypochlorite (NaOCl) followed by inactivation with sterile 5% sodium thiosulphate. The apical 5 mm of the root was sectioned perpendicular to the long axis of the tooth using a sterile carborundum disk. The cut surface of each root was cleansed and disinfected with NaOCl solution, which was then inactivated with sterile 5% sodium thiosulphate. Root canal samples were collected by introducing a sterile stainless steel K-file into the canal and applying a gentle filing motion. A small volume of sterile dH_2O was added into the canal and the sample was collected with a sterile paper point. The file and paper point were placed in 1.8 ml tubes containing 500 μl sterile dH_2O and immediately processed for DNA extraction.

DNA Extraction and PCR Amplification

The sample was dispersed by vortexing for 30 sec. File and paper point were removed, the microbial suspension was pelleted by centrifugation at $5,000\times g$ for 10 min. Total bacterial genomic DNA was isolated with the QIAamp DNA Micro Kit protocol to serve as template for amplification with the universal bacterial 16S rDNA primer pair "Bac1" that was fitted with a 40-bp GC clamp at the 5' end and "Bac2" (28).

Each 50 μl of PCR reaction contained 5 μl purified genomic DNA, 40 pmol of each PCR primers, 5 μl of $10\times\text{PCR}$ buffer, 0.2 mM dNTPs, 3.8 mM MgCl_2 , 1.0 U *Taq* DNA polymerase and was performed in a DNA thermocycler (BioRad). The cycling conditions were as follows: (i) an initial denaturation step at 94°C for 3 min; (ii) 30 cycles of a denaturation step at 94°C for 1 min, annealing step at 56°C for 1 min, and extension step at 72°C for 2 min; and (iii) a final extension step at 72°C for 5 min. Samples extracted from non-infected third molars served as controls. PCR amplification was confirmed by electrophoresis of 5 μl aliquots in a 1.0% agarose gel. The gel was stained with 0.5 $\mu\text{g/ml}$ of ethidium bromide and viewed under long-wavelength UV light.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE of PCR products was performed using the Bio-Rad DCode System (Hercules, CA). A 30% to 70% linear denaturing gradient (100% denaturant is equivalent to 7 M urea and 40% (v/v) deionized formamide) was formed in 8% (w/v) polyacrylamide gels and a 5 ml stacking gel without denaturant was added on top. Electrophoresis was performed at a constant 60 V at 60°C for 16 hrs in $1\times$ Tris-acetate-EDTA (TAE) buffer (pH 8.5). After electrophoresis, the gels were rinsed and stained for 15 min in $1\times$ TAE containing 0.5 $\mu\text{g/ml}$

ethidium bromide, followed by 15 min de-staining in 1× TAE buffer. DGGE profile images were digitally captured and recorded with the Molecular Imager Gel Documentation system (Bio-Rad Laboratories).

Analysis of DGGE Microbial Profiles

DGGE gel images were converted and transferred into a microbial profile database using Fingerprinting II Informatix™ Software (Bio-Rad). Each gel was normalized and the background subtracted with mathematical algorithms according to the spectral analysis of an overall densitometric curve. A 1.0% minimal profiling setting was employed for band search for all DGGE gels. Microbial diversity was assessed by measuring the total number of detected bands in each lane. The DGGE profiles were compared by pairwise similarity coefficient (C_s) analysis (18, 29) which considers the total number of bands in each of the compared samples as well as the number of bands found at the same position in the gel for both samples. C_s was calculated as follows: $C_s = 2j/(a+b) \times 100$ in which a and b designated the total number of bands present in samples a and b, respectively, and j was the number of bands found in both samples.

Sequencing and Sequence Analysis

PCR products were excised from the DGGE gel, eluted into 20 µl sterile dH₂O as previously described (18) and re-amplified with the Bac1/Bac2 universal primers. The resulting PCR products were purified and sequenced at the UCLA sequencing and genotyping core facility. The obtained partial 16S rRNA sequences were compared via BLAST with the HOMD, NCBI and RBPII databases. Sequences with 98 to 100% identity to sequences deposited in the public domain databases were considered to be positive identification of taxa.

RESULTS

The samples included in this study were collected from 15 patients with primary endodontic infections and 7 patients with secondary infections. Among the apical root portions sectioned from teeth with primary infection, five were obtained from the same patient and two of these were harvested from the mesial and distal roots of the same mandibular molar. Similarly, we collected the apical root portions from teeth with secondary infection from different patients, different teeth of the same patient (single rooted teeth) and different roots of the same teeth (mesial and distal root of the same mandibular molar as well as the palatal and disto-buccal root of the same maxillary molar). These samples allowed comparison of the pathogenic microbial profiles at various levels: a) type of endodontic infection (primary and secondary), b) inter-individual variability: unrelated exogenous microbiota (different individual), c) intra-individual variability: same exogenous microbiota but dissimilar local environment (different teeth of the same patient) and d) different locations within the same local environment (distinct roots of the same tooth).

All samples except those obtained from the uninfected control teeth yielded PCR products. Separation by DGGE produced an average of 33 ± 8 bands with a range of 16 to 50 and a median of 32 for primary infections (Figure 1A), while secondary infections (Figure 1B) contained significantly ($p < 0.0003$) fewer bands with an average of 16 ± 6 , a range of 9 to 26 and a median of 16.5. Sequencing allowed species/genus assignment to 20 of the bands present in either one or both types of infection (Table 1). Fusobacteria represented by either *Fusobacterium nucleatum* ssp. *animalis*, *Fusobacterium nucleatum* ssp. *nucleatum* or both were apparent in all primary infections (Figure 1A) and the majority of secondary infections (Figure 1B). Both subspecies produced one major band and two additional bands that became obvious when the species were more abundant. The generation of more than one band by certain species such as fusobacteria that contain multiple non-identical 16S rRNA

encoding genes has been reported before (29). *Actinomyces* sp. and *Anaeroglobus geminatus* were present in more than half of the samples for both types of infections. Similarly, *Pseudoramibacter alactolyticus* was detected in primary as well as secondary infections. *Porphyromonas endodontalis* and *Synergistetes* were found in the majority of primary infections. *Enterococcus faecalis*, a species long considered an important endodontic pathogen was present as a relatively faint band in less than half of the secondary infections. In the latter type of infection, putative environmental pathogens including *Pseudomonas* sp. as well as the *Burkholderiales* families *Burkholderia* and *Comamonadaceae* were apparent in the majority of samples. Additional species that were identified by sequencing are listed in Table 1.

Pairwise analysis of the individual banding patterns was employed to reveal the degree of similarity between all samples derived from the same type of infection. For primary infections, the following samples were available for analysis: 13 single rooted teeth from different patients (P1, P3 to P15), four teeth from the same patient (P2) which were comprised of three single rooted teeth (P2-2 to P2-4) and one mandibular molar for which the mesial and distal root samples were analyzed separately (P2-1a and P2-1b). Most of the samples obtained from the same patient (P2) had 50% or more similarity among each other (Figure 1A and Table 2 – P2-1a to P2-4). The microbial profiles for the two roots of the same tooth from this patient were very similar albeit not identical (Figure 1A and Table 2 – P2-1a and P2-1b). Some of the remaining samples from different patients exhibited the same degree of similarity as observed for those obtained from the same patients and even the same tooth (P2-1a/P2-1b vs P4/P5), while others had little in common (Figure 1A and Table 2).

The secondary infections exhibited equally distinct banding patterns between different patient samples (Figure 1B) that were comprised of four samples from single rooted teeth from different patients (S1 to S4), two different single rooted teeth from the same patient (S5-1 and S5-2) as well as four samples representing the mesial and distal root of a mandibular molar from one patient (S6-1a and S6-1b) as well as the palatal and disto-buccal root of a maxillary molar of a different patient (S7-1a and S7-1b). While the two apical portions from different teeth of the same patient harbored almost identical microbiotas (Figure 1B and Table 2, S5-1/S5-2), the two sets of samples derived from two roots of the same molar each displayed a surprising lack of similarity (Figure 1B and Table 2, S6-1a/S6-1b and S7-1a/S7-1b) in contrast to the comparable sample for the primary infections (Figure 1A, P2-1a/P2-1b). These disparities observed in different roots obtained from the same tooth with secondary infections included an apparent reduction of *Fusobacterium nucleatum* ssp *nucleatum* accompanied by an increase in *Burkholderiaceae* in one sample but not the other. At the same time the sample with more *Fusobacterium nucleatum* ssp *nucleatum* contained a noticeable higher amount of *Pseudomonas* sp. Another difference that was not correlated with the presence of fusobacteria was the absence of *Actinomyces* sp. in one root of the same tooth but not the other. Additionally, none of these samples contained *Anaeroglobus geminatus* which appeared to be present in most of the other samples with secondary infections.

DISCUSSION

In this study, we investigated the microbiota that colonizes the apical portion of root canals in primary and secondary infections. Ricucci *et al.* (30) demonstrated the presence of biofilm bacteria in this section of the root in the vast majority of cases with apical periodontitis. While successfully healed teeth often still contain bacteria in the coronal part of root canal, the apical sections are typically free of microorganisms (30). Even though the apical portion of infected root canals is considered most difficult to treat and thus most prone to harbor the residual infection (6, 24), few studies have performed separate analyses of the microbiota

located in this section of the tooth. Alves *et al.* (10) demonstrated that the bacterial profiles of the middle portion of teeth with primary and secondary endodontic infections differed significantly from the apical portion of the same teeth. A follow-up checker-board analysis examined these samples for the presence of a panel of 28 suspected endodontic pathogens (31). Two additional studies investigated the apical third of primary infected teeth for a number of species via species-specific PCR (14) and checker-board analysis (21).

We used a PCR-DGGE approach to obtain bacterial profiles for the apical portions of teeth derived from different individuals, different teeth of the same individuals as well as different roots of the same tooth. Both primary and secondary infections with chronic apical periodontitis were examined. While Alves *et al.* (10) found similar species diversity for both types of infections in similar samples, the primary infections analyzed here harbored significantly more diverse ($p=0.0003$) microbial profiles than the secondary infections (Figures 1A and 1B) with an average of 33 ± 8 vs 16 ± 6 bands, respectively. This observation is consistent with previous studies examining species diversity of the entire root canal (20, 23) and the difference to above study could be due to a number of reasons including geographical location (32, 33) and the smaller sample size examined by their group. Consistent with findings by other groups (18, 34), intra-individual and inter-individual variations were similar (Table 2). Collection of several pairs of apical portions derived from the same multi-rooted tooth allowed for the first time separate analysis of the species diversity present in the specific microenvironments of individual roots. Despite our small sample size it was apparent that the disturbance of the microbial biofilm during endodontic treatment produced conditions which resulted in a disparate recovery of the individual apical root-associated microbiota (Figure 1B – samples S6-1a/S6-1b and S7-1a/S7-1b). Among the identified microorganisms (Table 1), disparities were most obvious for *Fusobacterium nucleatum* ssp *nucleatum*, *Actinomyces* sp., *Pseudomonas* sp. and *Burkholderiales*. We believe that these differences in the respective microbiotas are most likely the result of incomplete eradication of the original infection. We propose that re-infection via coronal leakage should equally introduce a similar bacterial population to both roots and thus result in more comparable profiles as we observed for the two roots taken from the primary infected mandibular molar (Figure 1A – samples P2-1a/ P2-1b).

Phylotypic identification of over 50 excised bands revealed the high prevalence of fusobacteria, *Actinomyces* sp. and *Anaeroglobus geminatus* in both, primary and secondary infections. The suspected endodontic pathogen *Pseudoramibacter alactolyticus* (13–15,31) was also present in both types of infection (Table 1). Other species found in the majority of primary infections which were less abundant or not identified in previously treated teeth included *Porphyromonas endodontalis*, *Parascordavia denticolens*, *Prevotella* sp. *Dialister invisus*, and *Synergistetes*. Most of these species/taxa have been previously associated with endodontic disease (13–15,31). A striking observation in the samples from secondary infections was the high prevalence of *Pseudomonas* sp. as well as *Burkholderiales* which includes the families *Burkholderiaceae* and *Comamonadaceae* (Figure 1B and Table 1). These microorganisms are typically not abundant in the oral cavity and thus could indicate a problem with environmental contamination introduced during treatment. *E. faecalis* was present in less than half of the secondary infections as a relatively minor band consistent with previous observations that this species is unlikely a prime pathogen for apical periodontitis (9, 13,21,31,34,35).

In conclusion, the samples examined in our study exhibited a similar difference in bacterial profile diversity between primary and secondary infections in the apical root section as previously described for samples obtained from the entire root canal. In addition to environmental opportunistic pathogens, *Fusobacterium*, *Actinomyces* and *Anaeroglobus* are candidate genera for involvement in re-emergence/persistence of endodontic infections.

Examination of different roots from the same teeth indicated that disturbance of the microenvironment during treatment can lead to the development of divergent communities within the respective apical portions.

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Figure 1. Microbial community profiles of the apical portions of root canals with primary and secondary endodontic infections

(A) Microbial profiles of samples that were acquired from 15 patients with primary endodontic infections. The designation P1 to P15 refers to the different patients, while the additional label -1, -2 indicates that more than one tooth was obtained from a patient. The suffix a and b specifies that different roots of the same multi-rooted tooth were analyzed. Samples P1 and P3 to P15 thus represent the microbial profiles of single rooted teeth of different patients, while samples P2-1a to P2-4 were obtained from different teeth of the same patient. Samples P2-1a and P2-1b show the microbial profiles present in the mesial and distal root of the same mandibular molar. (B) Microbial profiles of samples that were

acquired from 7 patients with secondary endodontic infections. Similar to the samples presented in (A), the designation S1 to S7 refers to the individual patients, while –1, –2 indicates that more than one tooth was analyzed for a specific patient and a, b refers to the different roots of multirooted teeth. Samples S1 to S4 were obtained from single rooted teeth of different patients, S5-1 and S5-2 from two different single rooted teeth of the same patient. Microbial profiles shown in lanes S6-1a and S6-1b were obtained from the mesial and distal root of the same mandibular molar of patient #6, while lanes S7-1a and S7-1b reflect the microbial profiles of the palatal and disto-buccal root of the same maxillary molar of patient #7. The numbered bands were sequenced and correspond to the species listed in Table 1.

Table 1

Taxa identification of selected DGGE bands

Band	Taxon	Primary Infection (19 samples)	Secondary Infection (10 samples)
1	<i>Fusobacterium nucleatum</i> ssp. <i>animalis</i>	13	7
2	<i>Fusobacterium nucleatum</i> ssp. <i>nucleatum</i>	13	8
3	<i>Leptotrichia</i> sp.	p	n
4	<i>Lactobacillus</i> sp.	p	n
5	<i>Tannerella</i> sp.	n	p
6	<i>Porphyromonas endodontalis</i>	11	n
7	<i>Anaeroglobus geminatus</i>	9	5
8	<i>Pseudomonas</i> sp.	n	5
9	<i>Pseudoramibacter alactolyticus</i>	p	p
10	<i>Parascordavia denticolens</i>	p	n
11	<i>Dialister invisus</i>	p	n
12	<i>Enterococcus faecalis</i>	n	4
13	<i>Synergistetes</i>	11	n
14	<i>Atopobium</i> sp.	p	n
15	<i>Prevotella</i> sp.	p	n
16	<i>Oribacter</i> sp.	p	n
17	<i>Propionibacterium</i> sp.	n	4
18	<i>Comamonadaceae</i>	n	3
19	<i>Burkholderiales</i>	n	5
20	<i>Actinomyces</i> sp.	11	5

Numbers indicate how many samples contained a band running at the same location as the bands that led to identification of the species by sequencing.

p – species was identified in the samples that contained the corresponding sequenced band but tight banding pattern did not allow positive identification in samples in which bands were not sequenced

n– none of the bands sequenced in the indicated type of infection corresponded to this species

Table 2

C_S matrix for individual tooth samples

a) Primary infections																		
	P2 -1a	P2 -1b	P2 -2	P2 -3	P2 -4	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15
P1	58	62	36	51	61	38	49	56	32	45	29	46	43	50	43	24	42	62
P2-1a		89	51	56	60	56	53	53	51	50	45	51	55	60	41	30	56	63
P2-1b			56	57	65	61	58	54	49	45	40	46	59	67	47	40	61	65
P2-2				41	44	33	31	42	30	12	37	36	26	35	42	21	33	42
P2-3					64	25	50	42	21	46	32	28	39	35	29	16	29	43
P2-4						30	56	55	31	44	42	41	42	60	46	23	33	60
P3							52	42	41	34	21	15	43	43	29	34	48	39
P4								89	44	41	23	29	50	63	32	42	22	49
P5									45	48	31	42	50	55	54	39	55	59
P6										33	21	18	38	35	28	53	57	38
P7											43	42	46	46	38	26	31	32
P8												52	58	44	34	6	21	39
P9													47	54	16	14	19	46
P10														61	40	27	36	47
P11															44	24	44	43
P12																24	42	47
P13																	64	23
P14																		46

b) Secondary infections									
	S2	S3	S4	S5 -1	S5 -2	S6 -1a	S6 -1b	S7 -1a	S7 -1b
S1	47	55	41	54	57	53	39	58	48
S2		35	32	30	40	34	19	50	44
S3			27	47	57	42	33	39	29
S4				23	17	47	40	22	32
S5-1					81	52	14	51	41
S5-2						55	23	48	38

b) Secondary infections

	S2	S3	S4	S5 -1	S5 -2	S6 -1a	S6 -1b	S7 -1a	S7 -1b
S6-1a						55	51	51	51
S6-1b							30	42	42
S7-1a								55	55

P1 to P15 indicate different subjects that contributed the teeth with primary infections examined in this study. For subjects that contributed more than one tooth (boxed gray shaded samples with the individual teeth separated by dashed lines) the suffix -1, -2 etc indicated the different teeth obtained. Samples from different roots of the same tooth are labeled with the additional suffix a or b and highlighted in a darker shade of gray.

S1 to S7 indicate the different subjects that contributed the teeth with secondary infections examined in this study. For additional information see legend table 1a.