Short Communication

Characterization of bacterial flora in persistent apical periodontitis lesions

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Abstract

Introduction: Microorganisms are able to survive and induce persistent infection in periapical tissues. The aim of this study was to investigate the composition of the microflora of persistent apical periodontitis lesions.

Methods: Twenty-one apical lesion samples were obtained from 21 patients with chronic apical periodontitis by root end surgery and processed using aerobic or anaerobic culture techniques. All isolated strains were identified by 16S rDNA sequence analysis.

Results: Seventy-four strains were isolated, belonging to 31 bacterial species obtained from the 20 apical lesions were isolated. The majority of the strains were facultative anaerobes (51.6%). *Propionibacterium acnes, Staphylococcus epidermidis, P. aeruginosa* and *Fusobacterium nucleatum* were isolated in 16.2%, 9.5%, 6.8% and 5.4% of the samples, respectively. Fifteen samples harboured more than one species. The predominant association was *Propionibacterium acnes, Staphylococcus epidermidis* and *Fusobacterium nucleatum.*

Conclusion: The microbiota of persistent apical periodontitis lesions is composed by diverse types of microorganism with biofilm-forming capacity including *P. acnes, S. epidermidis* and *F. nucleatum*.

Apical periodontitis is mainly caused by microorganisms originating from the root canal (37). Presence of bacteria is associated with non-healing apical periodontitis (14). Therefore, the elimination of microorganisms from the root canal system is crucial in resolving apical periodontitis (32). However, the complexity of the root canal system makes their complete elimination difficult.

In persistent periodontitis, microorganisms form a biofilm consisting of a mixed population (24, 35). Microorganisms in biofilm possess characteristics that differ from their planktonic form, including resistance to phagocytic cells and drugs, resulting in persistent infection (11). A relationship between specific microorganisms and type of apical periodontitis has been reported (4, 40). However, the identity of the specific species involved in persistent periodontitis remains to be clarified. The aim of this study was to investigate the biofilm-forming bacterial flora in persistent periodontitis lesions using 16S rDNA bacterial identification.

Twenty-three patients (14 men and 9 women, mean age 46.0 years) attending the Tokyo Dental College Chiba Hospital were enrolled in the study. Informed consent was obtained from all patients. No patient had systemic disease or received antibiotic

therapy during the 3-month period leading up to root canal treatment. All patients were diagnosed with chronic apical periodontitis requiring root end surgery. All procedures conformed to the protocols approved by the Institutional Ethical Review Board of Tokyo Dental College. Twenty samples from chronic apical periodontitis lesions were obtained from the apices of teeth in which had received nonsurgical root canal treatment and obturation had been carried out. The 20 apical samples consisted of 19 incisors and one molar. The concomitant presence of a peripheral radiolucent area and no root fracture or periodontal pocket formation at the root apex were observed in all 20 teeth. Eight of the 20 lesions had sinus tracts. Based on lesion size and obturation status, the teeth were treated with root end surgery.

Apex samples were obtained directly from apical lesions during root end surgery. After applying local anesthesia, the operative field was washed thoroughly with 7.5% povidone-iodine solution. Following marginal incision, full-thickness flaps were elevated. Access to root apex lesions was achieved with a low-speed handpiece equipped with a sterilized tungsten-carbide round bur under application of sterilized phosphate buffered saline (pH 7.4; PBS, Nissui Pharmaceutical, Tokyo, Japan) for cooling. After exposure of the apex, an apical 3 mm of the root was resected perpendicularly to the long axis of the tooth with a sterilized tapered diamond bur in a

high-speed handpiece under sterilized PBS cooling. These sections were used as samples for isolation of microorganisms. All procedures were performed in a manner that avoided salivary bacterial contamination or exposure to air for a protracted period of time.

Isolated root apices were immediately transferred to a sterile vial containing 900 μl reduced transport fluid (RTF) (38) and sterile glass beads. The microorganisms on the surface of each apex were then dispersed with a vortex mixer for 5 min. The suspension was then serially diluted from 10^{-1} to 10^{-5} with RTF. Each 100 μl diluted suspension was inoculated onto Tryptic soy agar (Becton Dickinson Microbiology System, Cockeysville, MD) plates containing 5 μg/ml hemin, 0.5 μg/ml menadione and 10% horse blood, followed by incubation either anaerobically or aerobically for one week. Each distinct colonial type from both cultures was subcultured repeatedly for purity. The purity of each isolate was confirmed by colony morphology and cellular shape following Gram-staining.

Genomic DNA from each isolated strain was analyzed using the Puregene DNA Purification Kit (Genetra Systems, Inc., Minneapolis, MN, USA) according to the supplier's instructions. Briefly, approximately 1.0 x 10^9 bacterial cells were lysed with 300 μl Cell Lysis Solution and samples incubated at 80^{o}C for 5 min. After treatment

with RNase A, 100 μl Protein Precipitation Solution was added and supernatant obtained by centrifugation. Bacterial DNA was precipitated with 300 μl 100% isopropanol. For all isolates, the 16S RNA coding sequence was amplified with the MicroSeq Full Gene 16S rDNA Bacterial Identification Kit (Applied Biosystems, Foster City, CA, USA) according to the instructions supplied. Briefly, three regions of 16s rRNA coding sequence were amplified with the primer supplied. The PCR products were then sequenced with primers included in the kit and the 310 Genetic Analyzer (Applied Biosystems). The obtained sequences were then compared with the 16S rRNA coding locus in the public sequence database (GeneBank) and species containing identical sequences identified.

All samples yielded positive microbial growth. A total of 74 strains were isolated. Sequencing of 16S rDNA of these strains revealed that these strains belonged to 31 bacterial species. Isolated species are listed in Table 1. The strains isolated consisted of facultative anaerobic bacteria (51.6%), obligate anaerobic bacteria (38.7%) and aerobic bacteria (9.7%). Gram-positive cocci, Gram-positive rods, Gram-negative cocci and Gram-negative rods accounted for 32.3%, 19.4%, 3.2% and 45.1% of the samples, respectively. The predominant genera were *Staphylococcus*, *Propionibacterium*, *Prevotella*, *Streptococcus*, *Fusobacteirum* and *Pseudomona*s.

Four of the 5 *P. aeruginosa* and all of the *Klebsiella pneumoniae* strains were detected from sinus tract-forming apical lesions. Eight of the 12 *P. acnes*, 4 of the 7 *S. epidermidis* and all of the *F. nucleatum* strains were detected from non-sinus tract-forming apical lesions. We detected both aerobic and anaerobic species in 3 apical lesions. Two of the lesions were exposed to the mouth by sinus tract.

Combinations of mixed infections from 20 apical periodontitis lesions are listed in Table 2. Monoinfection with either *P. acnes* or *P. aeruginosa* was detected in two apical lesions, respectively. *Staphylococcus cohnii* was isolated from one other monoinfected lesion. Two to 8 bacterial species were isolated from 15 lesions from the 20 apical periodontitis samples. *P. acnes*, *S. epidermidis* and *F. nucleatum* were identified most frequently in multiple infections consisting of two or three bacterial species.

The detection profile of multi-bacterial species in this study agrees with that in previous reports on apical periodontitis and abscesses (7, 15, 25). The results of the present study and those of previous studies (6, 22, 35) revealed a mixture of obligate anaerobes and facultative anaerobes in the microflora in chronic apical periodontitis. A combination of obligate and facultative anaerobes was shown to be predominant in most types of periapical abscess and odontogenic infection (5, 18). Noguchi et al (23)

investigated the microflora in persistent periodontitis and reported frequent detection of Gram-negative obligate anaerobes, including *F. nucleatum*. This microorganisms also frequently isolated from root-filled teeth with peridadicular lesions (30). *F. nucleatum* coaggregates with many species of dental plaque bacteria and plays an important role in biofilm formation (17). Biofilm is involved in persistent infection in human (11). Decrease in susceptibility to antibiotics and increase in resistance to phagocytic cells have been reported in a number of microorganisms in biofilm (8, 13, 39). Several reports have shown biofilm formation at the apex (19, 23, 24). Enhanced of attachment of *Porphyromonas gingivalis* to human fibroblasts by *F. nucleatum* was reported (20). One of the isolates in the present study, *F. nucleatum* strain TDC100, showed a synergistic effect on biofilm formation with *P. gingivalis* (28). In addition, *F. nucleatum* TDC100 enhanced invasion of human epithelial and aortic endothelial cells by *P. gingivalis* (27) .

S. epidermidis has been isolated from persistent periodontitis and/or dentoalveolar abscesses (33, 41). We also isolated this species at high frequency from apical lesions. This species has also been reported to form biofilm (8). In an earlier study, we observed a synergistic effect between *S. epidermidis* TDC78 and *F. nucleatum* TDC100 on biofilm formation, and both those strains were obtained in this study (28).

Although frequently isolated in persistent periapical periodontitis lesions, *E. faecalis* was not detected in the present study. *E. faecalis* is the most commonly found species in root canal-treated teeth exhibiting persistent disease (31, 36). However, Sakamoto et al reported that new candidate endodontic pathogens, including as-yet-uncultivated bacteria and taxa other than *E. faecalis*, may participate in mixed infections associated with post-treatment apical periodontitis (29). Further analysis should be required in order to clarify the reason for this discrepancy, taking into account treatment history and condition of patients.

In the present study, we frequently isolated *S. epidermidis, P. acnces* and *P. aeruginosa.* We also isolated both *S. cohnii* and *S. warneri,* although they are not frequently isolated in human infections (2). These microorganisms are not considered major members of the oral microflora in human. However, frequent recovery of *P. acnes, P aeruginosa* and *S. epidermidis* was also reported previously (1, 9, 21). These three reports analyzed apical lesions of obturated teeth. *S. worneri, S. capitis* and *S. hominis* were isolated from dental plaque, saliva and nasal swab (26). *S. cohnii* and *S. pasteurii* were detected from the oral cavity (3, 16). Although it is possible that this was due to contamination from the environment during sampling, level of staphylococci in saliva was 10^2-10^4, whereas that of streptococci was approximately

10^{7-8}(26, 34). In the present study, detection rate of streptococci was low. This suggests that these staphylococci were not isolated due to contamination. This unique bacterial profile may have been due to the site of the lesions, which were developed at the apex after obturation, although further investigation is required to confirm this.

We identified *P. acnes* strains at a relatively high frequency in samples of periapical lesions. The high detection of Propionibacterium species from periapical lesions was also demonstrated as described above (1). *P. acnes* strains coaggregated with *Streptococcus sanguinis* (10). This suggests the ability of *P. acnes* strains to form polymicrobial biofilm with other bacterial species. It is possible that *P. acnes* forms biofilm by coaggregating with other previously attached microorganisms such as *S. sanguinis.*

P. aeruginosa is known to produce alginic extracellular polymeric substances and form persistent biofilm, and is also resistant to chemotherapy (11, 12). This microorganism was also detected from root canal treated teeth with radiolucent lesions (9). The resistance of this microorganism to antibiotics, as well as its biofilm-forming ability, may contribute to the development of persistent periapical lesions.

Taken together with those of previous studies, the results of the present study indicate that biofilm-forming microorganisms such as *P. acnes, S. epidermidis, P.*

aeruginosa and *F. nucleatum* are involved in the development of persistent apical lesions.

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Table 1. Bacterial species isolated from apical periodontitis lesions of obturated teeth

Bacterial species	No. of samples		
	Total (% of isolates)	Sinus tract	
		+	-
Staphylococcus epidermidis	7 (9.5)	3	4
Staphylococcus warneri	1 (1.4)	0	1
Staphylococcus capitis	3 (4.1)	1	2
Staphylococcus hominis	1 (1.4)	1	0
Staphylococcus pasteuri	1 (1.4)	1	0
Staphylococcus cohnii	1 (1.4)	1	0
Streptococcus sanguinis	2 (2.7)	1	1
Streptococcus parasanguis	1 (1.4)	0	1
Streptococcus species*	1 (1.4)	0	1
Slackia exigua	1 (1.4)	0	1
Peptostreptococcus micros	3 (4.1)	1	2
Bacillus licheniformis	1 (1.4)	1	0
Corynebacterium simulans	1 (1.4)	0	1

Propionibacterium acidipropionici	1 (1.4)	0	1
Propionibacterium acnes	12 (16.2)	4	8
Actinomyces naeslundii	2 (2.7)	0	2
Klebsiella pneumoniae	2 (2.7)	2	0
Veillonella atypica	1 (1.4)	0	1
Stenotrophomonas maltophilia	1 (1.4)	0	1
Dialister invisus	1 (1.4)	1	0
Porphyromonas gingivalis	2 (2.7)	1	1
Prevotella dentalis	2 (2.7)	2	0
Prevotella buccae	1 (1.4)	0	1
Prevotella nigrescens	1 (1.4)	0	1
Prevotella loescheii	1 (1.4)	0	1
Prevotella enoeca	1 (1.4)	1	0
Fusobacterium nucleatum	4 (5.4)	0	4
Fusobacterium naviforme	1 (1.4)	0	1
Pseudomonas aeruginosa	5 (6.8)	4	1
Roseomonas mucosa	2 (2.7)	1	1
Campylobacter rectus	1 (1.4)	0	1

* 16S rRNA sequence showed high homology with Streptococcus genomospecies.

Table 2. Identified bacterial species and combinations of mixed infection lesions with apical periodontitis

Case no.	Bacteria
Without sinus tract	
1	*Streptococcus parasanguis, Staphylococcus epidermidis, Staphylococcus capitis, Slackia exigua, Fusobacterium nucleatum, Actinomyces naeslundii*
2	*Porphyromonas gingivalis, Streptococcus sanguinis, Propionibacterium acidipropionici, Prevotella loescheii, Propionibacterium acnes, Peptostreptococcus micros*
3	*Staphylococcus epidermidis, Propionibacterium acnes*
4	*Propionibacterium acnes*
6	*Propionibacterium acnes*
7	*Staphylococcus epidermidis, Streptococcus warneri*
10	*Propionibacterium acnes, Pseudomonas aeruginosa, Campylobacter rectus*
12	*Propionibacterium acnes, Staphylococcus epidermidis, Fusobacterium nucleatum, Staphylococcus capitis, Roseomonas mucosa*
13	*Fusobacterium naviforme, Prevotella buccae, Peptostreptococcus micros*
14	*Propionibacterium acnes, Veillonella atypica*
15	*Fusobacterium nucleatum, Propionibacterium acnes, Corynebacterium simulans*
16	*Stenotrophomonas maltophilia, Streptococcus* species* *Actinomyces naeslundii, Prevotella nigrescens*
With sinus tract	
5	*Staphylococcus cohnii*
8	*Propionibacterium acnes, Staphylococcus epidermidis, Prophylomonas gingivalis, Bacillus licheniformis*

9	*Staphylococcus hominis, Staphylococcus epidermidis, Staphylococcus pasteuri, Pseudomonas aeruginosa, Prevotella dentalis, Prevotella enoeca, Propionibacterium acnes, Dialister invisus*
11	*Propionibacterium acnes, Staphylococcus epidermidis, Staphylococcus capitis, Roseomonas mucosa, Fusobacterium nucleatum, Pseudomonas aeruginosa, Prevotella dentalis*
17	*Klebsiella pneumoniae, Peptostreptococcus micros*
18	*Klebsiella pneumoniae, Propionibacterium acnes, Streptococcus sanguinis*
19	*Pseudomonas aeruginosa*
20	*Pseudomonas aeruginosa*

* 16S rRNA sequence showed high homology with Streptococcus genomospecies.