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Short communication

Characterization of bacterial flora in persistent apical periodontitis lesions

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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 apical lesion samples were obtained from 20 patients with chronic apical periodontitis by root end surgery and processed using aerobic or anaerobic culture techniques. All isolated strains were identified by 16S ribosomal DNA sequence analysis.
Results: Seventy-four strains were isolated, belonging to 31 bacterial species obtained from the 20 apical lesions that were isolated. The majority of the strains were facultative anaerobes (51.6%). *Propionibacterium acnes*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Fusobacterium nucleatum* were isolated from 16.2, 9.5, 6.8 and 5.4% of the samples, respectively. Fifteen samples harboured more than one species. The predominant association was *P. acnes*, *S. epidermidis* and *F. nucleatum.*
Conclusion: The microbiota of persistent apical periodontitis lesions is composed by diverse types of microorganisms with biofilm-forming capacity, including *P. acnes*, *S. epidermidis* and *F. nucleatum*.

Key words: apical periodontitis; bacteria; bacterial consortia; biofilm; persistent periodontitis; polymicrobial infection

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Apical periodontitis is mainly caused by microorganisms originating from the root canal (37). The 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 forms, 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 ribosomal DNA (rDNA) bacterial identification.

Twenty-three patients (14 men and nine 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 that had received non-surgical root canal treatment and in which 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 (PBS; pH 7.4; Nissui Pharmaceutical, Tokyo, Japan) for cooling. After exposure of the apex, the apical 3 mm of the root was resected perpendicular 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 (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 reduced transport fluid. 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 1 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 analysed using the Puregene DNA Purification kit (Genetra Systems, Inc., Minneapolis, MN) according to the supplier's instructions. Briefly, approximately 1.0×10^9 bacterial cells were lysed with 300 μl Cell Lysis Solution and samples were incubated at 80°C for 5 min. After treatment with RNase A, 100 μl Protein Precipitation Solution was added and the supernatant was obtained by centrifugation. Bacterial DNA was precipitated with 300 μl 100% isopropanol. For all isolates, the 16S ribosomal RNA (rRNA) coding sequence was amplified with the MicroSeq Full Gene 16S rDNA Bacterial Identification kit (Applied Biosystems, Foster City, CA) according to the instructions supplied. Briefly, three regions of 16S rRNA coding sequence were amplified with the primer supplied. The polymerase chain reaction (PCR) products were then sequenced with primers included in the kit and the 310 Genetic Analyzer (Applied Biosystems). The sequences obtained were then compared with the 16S rRNA coding locus in the public sequence database (GenBank) 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 five *Pseudomonas aeruginosa* and all of the *Klebsiella pneumoniae* strains were detected from sinus tract-forming apical lesions. Eight of the 12 *Propionibacterium acnes*, four of the seven *Staphylococcus epidermidis* and all of the *Fusobacterium nucleatum* strains were detected from non-sinus tract-forming apical lesions. We detected both aerobic and anaerobic species in three 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 eight 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 multibacterial 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

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

	No. of samples		
		Sinus tract	
Bacterial species	Total (% of isolates)	+	–
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 (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

[1]16S ribosomal RNA 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,[1] *Actinomyces naeslundii*, *Prevotella nigrescens*
With sinus tract	
5	*Staphylococcus cohnii*
8	*Propionibacterium acnes*, *Staphylococcus epidermidis*, *Porphyromonas 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*

[1]16S ribosomal RNA sequence showed high homology with *Streptococcus* genomospecies.

reported frequent detection of gram-negative obligate anaerobes, including *F. nucleatum*. These microorganisms were 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 humans (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 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 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, *Enterococcus 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 is required 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. acnes* and *P. aeruginosa*. We also isolated both *S. cohnii* and *Staphylococcus warneri*, although they are not frequently isolated in human infections (2). These microorganisms are not considered major members of the oral microflora in humans. However, frequent recovery of *P. acnes*, *P. aeruginosa* and *S. epidermidis* was also reported previously (1, 9, 21). These three reports analysed apical lesions of obturated teeth. *S. warneri*, *Staphylococcus capitis* and *Staphylococcus hominis* were isolated from dental plaque, saliva and nasal swab (26). *S. cohnii* and *Staphylococcus pasteurii* were detected from the oral cavity (3, 16). Although it is possible that this was the result of contamination from the environment during sampling, the level of staphylococci in saliva was 10^2–10^4, whereas that of streptococci was approximately 10^7–10^8 (26, 34). In the present study, the detection rate of streptococci was low. This suggests that these staphylococci were not isolated as the result of 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 biofilms 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 biofilms, 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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