Status of bacterial colonization in teeth associated with different types of pulpal and periradicular disease: A scanning electron microscopy analysis

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Abstract  Background/purpose: The purpose of this study was to use scanning electron microscopy (SEM) to investigate the status of bacterial colonization in differently infected root canals and the damage to radicular dentin.  Materials and methods: Twenty-five freshly extracted teeth were selected for this study (Group A: 8 teeth with pulpitis; Group B: 10 teeth with periapical lesions; and Group C: 7 teeth with failed root canal treatment). After fixation, the teeth were longitudinally split into two halves. The halves were then dehydrated, sputter-coated with gold, and viewed using SEM, descriptively dividing their lengths into apical, middle, and coronal thirds.  Results: In Group A, bacterial infection was mainly located in the coronal third of the root canals and bacteria failed to penetrate into the dentinal tubules. In Group B, bacterial infection was distributed over the entire length of the root canal. The invasion depth of bacteria into the dentinal tubules was approximately 300 μm. In Group C, bacterial infection was mainly focused on the apical third of the root canals. Most of the dentinal tubules had collapsed, and the root canal walls were heavily colonized with dense bacterial biofilm, primarily consisting of cocci. Compared to Group B, the invasion depths were deeper in the apical thirds of root canals (P < 0.05).  Conclusion: Bacterial infection was lighter in the root canals with pulpitis than in those with apical periodontitis, which might require special considerations regarding different stages of pulp and periapical pathology in root canal treatment.

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Introduction

Pulpal and periradicular disease is one of the most common infectious diseases threatening human health. Bacteria, usually from dental caries, are the major etiological agents of pulp and periradicular disease. Related research as previously described has indicated that infected root canals act as habitats for bacteria, which exist in planktonic forms, aggregates, and coaggregates and in the biofilm status in complex communities that are composed of bacterial cells and extracellular matrix. Root canal therapy (RCT) is the most commonly applied, effective method in clinical treatment for pulp and periradicular disease. The goal of treatment of the disease has been total eradication from the infected root canal systems and prevention of reinfection. According to statistics, RCT has failure rates between 4% and 15%, whereas the rate of successful root canal retreatment was approximately 74%. The most important factor in endodontic failure is the incomplete eradication of bacteria. With improved bacterial culture and analysis techniques, intracanal bacteria have been extensively studied and exactly classified. However, these investigations have not provided sufficient information concerning the bacterial colonization status in the dentinal wall of different types of pulpal and periradicular disease. Knowledge of the status of bacterial colonization could provide us with a better understanding of the disease process. Scanning electron microscopy (SEM) has excellent resolution and can reveal details regarding the bacterial colonization status in natural environments. Therefore, this study sought to characterize the process of disease and to provide a theoretical basis for the establishment of effective treatment strategies using SEM to examine the bacterial colonization of infected root canals associated with different types of pulpal and periradicular disease. Thus, it can provide us with clinical guidance.

Materials and methods

The study protocol was reviewed and approved by the Ethics Committee of Nanjing University Stomatological Hospital (Nanjing, China). Verbal and written consent was obtained from all of the study participants prior to extraction of teeth.

Patients and specimen collection

The examined material consisted of 25 extracted teeth randomly collected from 25 patients at Nanjing University Stomatological Hospital. Healthy adult volunteers were age 18–70 years. A detailed medical and dental history was obtained from each patient. All patients were in good health and were not taking any medication that would alter bacteria status during the past 3 months. Teeth with periodontitis or fractures were excluded from the study. Teeth were extracted for reasons not related to this study. All samples for the pulpitis group were from the third molars. For primary and secondary apical periodontitis, we collected samples from these cases as the third molars, those that could not meet with the lowest conditions for oral rehabilitation, and the extracted cases for personal reasons.

The extracted teeth were divided into three groups according to the clinical and radiographic examination: Group A (8 teeth): pulpitis; Group B (10 teeth): primary apical periodontitis; and Group C (7 teeth): secondary apical periodontitis.

The inclusion criteria were as follows. Group A: Pulpitis is characterized by a history of provoked and spontaneous dull, heavy, and lingering thermal pain that can be reproduced clinically. Radiographs show the depth of the caries or cavity preparation. The periodontal ligament space and lamina dura are normal. Group B: Presence of clinical signs and symptoms of chronic apical periodontitis (pain, swelling, or sinus tract); no previous endodontic treatment; and diameter of the periapical radiolucent area of at least 3 mm. Group C: Endodontic treatment performed for > 2 years and radiographically visible filling of root canal: (1) the presence of obvious clinical signs and symptoms (pain on palpation, discomfort to percussion, and pain of the sinus tract); and (2) the persistent or emergent periapical radiolucency. The appearance of one of these two items is considered to be a treatment failure.

Sampling procedures

After disinfection of the tooth crown and the adjacent tissues with 2% chlorhexidine digluconate solution, the tooth was carefully extracted. Subsequently, the periodontal ligament and other attachments were removed with a scalpel, and the clinical crown was sectioned at the cementoenamel junction with carborundum disks. Then, the teeth were immediately immersed in 2.5% phosphate-buffered glutaraldehyde solution. The sample teeth were stored at 4°C to provide a total fixation period of 1 week. After fixation, longitudinal grooves (approximately 2 mm) were cut along the entire root length with tapered diamond burs under a water spray. The roots were then split with a chisel into two halves. The root canal length was measured and divided equally into coronal, middle, and apical thirds, which were marked with a scalpel blade.

SEM preparation and observation

Each root half was then gently washed in phosphate buffered saline (pH = 7.2, 4°C), dehydrated in increasing concentrations of ethanol (50%, 60%, 70%, 80%, 90%, and 2 × 100% for 15 minutes each), critical-point dried using liquid CO2 replacement, coated with gold, and imaged in a scanning electron microscope (S-3400N, Hitachi, Tokyo, Japan) at a voltage of 20 kV. Thereafter, the entire root half, the radicular dentinal wall, and the dentinal tubules were examined from low to high magnification using SEM. Observation was conducted, and photographic images were obtained.

Statistical analysis

Statistical evaluation of the study results was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA) and...
using the Chi-square test. A P value of <0.05 was considered to be statistically significant.

**Results**

The longitudinal distribution of bacterial cells in teeth associated with different types of pulpal and periapical disease is outlined in Table 1.

In the coronal third of the root canals, the positive number of bacterial colonization was in six of eight specimens in Group A, eight of 10 specimens in Group B, and four of seven specimens in Group C. No significant differences were observed between the three groups. In the middle third of the root canals, the statistical analysis revealed that the bacterial detection rate was much lower in Group A (1/8, 13%) than in Group B (9/10, 90%) or Group C (4/7, 57%). However, no significant differences were observed between Group B and Group C. In the apical third of the root canals, bacteria were present in all of the examined specimens in Group B and Group C, whereas the situation in Group A (no bacteria were observed in Group A) was the opposite.

The results also revealed that bacterial infection with pulpitis was mainly confined to the crown (P < 0.05), whereas bacterial infection with primary apical periodontitis was distributed over the entire root half (P > 0.05). For secondary apical periodontitis, 100% of the apical thirds exhibited bacterial infection, but only approximately 50% of the specimens were infected in the coronal and middle thirds (P > 0.05).

A comparison of the invasion depth of bacteria into dentinal tubules in the middle and apical thirds of primary and secondary apical periodontitis is presented in Table 2.

In the middle third of the root canals, the invasion depth of bacteria into the dentinal wall was >300 μm in 34% of the specimens in Group B, whereas the invasion depth was <300 μm in all of the specimens in Group C. However, no significant differences were observed in the situation (P > 0.05). In the apical third of the root canals, the invasion depth of bacteria into the dentinal wall was >300 μm in 86% of the specimens in Group C, whereas the invasion depth was <300 μm in 80% of the specimens in Group B, which had significant differences between Group B and Group C (P < 0.05).

The patterns of bacterial colonization and dentinal structural changes of different types of pulpal and periapical disease are described in Figs. 1–3.

In the infected root canals with pulpitis, a small amount of bacterial aggregates was present in the root canal walls and lumen, and the invasion depth was <100 μm in the coronal area (Fig. 1A and B). In the middle and apical thirds, no bacterial colonization or invasion of the dentinal tubules was observed, but erythrocytes were visible in the apical area (Fig. 1C–F).

In the coronal thirds of infected root canals with primary apical periodontitis, a large number of amorphous materials and bacterial aggregates were observed in the root canal lumen (Fig. 2A). In addition, bacterial aggregates were present on the dentinal walls and in the dentinal tubules (Fig. 2B). In the middle third, amorphous materials and bacterial aggregates were present in larger amounts, and the bacterial invasion depth was >300 μm (Fig. 2C and D). In the apical third, a large number of bacterial aggregates had penetrated into the dentinal tubules to approximately 300 μm, and bacteria were colonized more heavily in the shallow dentinal tubules than in the deep dentinal tubules (Fig. 2E and F).

In the coronal and middle third areas of the infected root canals with secondary apical periodontitis, amorphous materials and bacterial aggregates consisting of cocci and rods adhered to the root canal walls, colonized the dentinal surface, and progressed toward the dentinal tubules (Fig. 3A–D). In the apical area, most of the dentinal tubules had collapsed, and the root canal walls were heavily colonized with a dense bacterial biofilm consisting of cocci, rods, or filaments (Fig. 3E and F).

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**Table 1** Comparison of positive rates of bacterial detection in all root canal segments.a

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Positive rates of bacterial detection (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Crown third</td>
<td>Middle third</td>
</tr>
<tr>
<td>A</td>
<td>8</td>
<td>6 (75)</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>8 (80)</td>
</tr>
<tr>
<td>C</td>
<td>7</td>
<td>4 (57)</td>
</tr>
</tbody>
</table>

Data are presented as n (%).

* *P* < 0.05 compared with Group A.

**P** < 0.05 compared with the coronal third.

a The bacteria or bacterial aggregates for areas of each root canal part were observed by scanning electron microscopy under >600 magnification. If we found more than five bacteria or bacterial aggregates, we defined this root canal part as having positive results for bacterial colonization.

**Table 2** Comparison of the invasion depths of bacteria into the dentinal walls in the middle and apical thirds of the root canals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Extent of the different invasion depths (%)a</th>
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<tbody>
<tr>
<td></td>
<td>Number</td>
</tr>
<tr>
<td></td>
<td>&lt;100 μm</td>
</tr>
<tr>
<td>B</td>
<td>9</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
</tr>
<tr>
<td>P</td>
<td>&gt;0.05</td>
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</tbody>
</table>

Data are presented as n (%).

a Invasion depth: the perpendicular distance from the deepest bacterial aggregate to the root canal dentinal wall.
Figure 1  (A–F) Scanning electron microscopy of bacterial colonization with pulpitis. Coronal third portion: (A) a small amount of bacteria in the root canal lumen (×1000, red arrows). (B) The invasion depths were <100 μm (×500); inset is ×5000 magnification. Middle and apical third portions: (C and E) no bacterial colonization, but erythrocytes were visible in the root canal lumen (×3000, ×2000, red arrows); (D and F) no bacterial invasion into the dentinal tubules (×500, ×500); inset is ×5000 magnification.

Figure 2  (A–F) Scanning electron microscopy of bacterial colonization with primary apical periodontitis. Coronal third portion: (A) a large number of amorphous materials and bacterial aggregates in the root canal lumen (×1000, red arrows); (B) bacterial aggregates on the dentinal walls and in the dentinal tubules (×600); inset is ×30,000 magnification. Middle and apical third portions: (C and E) amorphous materials and bacterial aggregates were present in larger amounts (×1000, ×1000, red arrows); (D and F) bacterial invasion depth was equal to or greater than 300 μm (×300, ×1000); inset is ×5000 magnification.
Discussion

To date, some studies of the location and distribution of bacterial colonization in infected root canals have been published, but the infected root canals have rarely been classified according to different stages of pulp and periapical pathology in these studies. Therefore, in our current research, we classified the infected root canals into the following three groups: pulpitis, primary apical periodontitis, and secondary apical periodontitis. Richardson et al., using different microscopy techniques [light microscopy (LM), transmission electron microscopy (TEM), and SEM] and protocols (Eastman Dental Institute, London, Britain and Zurich, Switzerland), found that microorganisms organized into biofilm mainly on the root canal wall in the apical third. Noiri et al. found that bacteria were non-uniformly distributed in the same infected root canal, and these bacteria were almost in the planktonic status in the coronal and middle thirds but formed typical biofilm in the apical third. The observations in this study demonstrated that there was no bacterial biofilm detected in the teeth with primary apical periodontitis and the bacteria in the teeth with failed RCT (secondary apical periodontitis) were mainly organized as biofilms in the apical third, which differed from the results of Richardson et al. and Noiri et al., who did not classify apical periodontitis into primary and secondary types. As a result, they did not definitively indicate whether the visualized biofilm adhering to the root canal wall was in teeth with periapical lesions or failed RCT. Biofilms are 1000 times more resistant to antimicrobial medications than the same bacteria in the planktonic state because biofilms can tolerate the harsh intracanal environment and can multiply rapidly whenever feasible, causing intracanal reinfection, damage to the periapical tissues, and eventual endodontic failure, which is not difficult to explain earlier findings that long-term residual bacteria in root canals after obturation were a major cause of treatment failure.

In in vitro studies, the invasion depth of bacteria into the test tube could reach 900 µm, mainly because there was no smear layer in the test tube. Such invasion depth was not ever reported in tooth specimens. Peters et al. found that microbes could invade dentine tubules up to 500 µm or even deeper. This study concluded that bacteria primarily found in the apical third segment could invade the dentine tubules to varying depths between 100 µm and 300 µm. In fact, the permeability of root canal irrigation drugs is limited. For example, the permeability of sodium hypochlorite was only approximately 130 µm into the dentine tubules. During the process of root canal cleaning and shaping, bacteria in the root canal lumen and shallow dentinal walls were obviously eliminated as a result of the function of irrigation drugs and the removal of diseased dentin. However, deep-seated bacteria in the dentinal tubules are difficult to remove absolutely. For this reason, ultrasound flushing is recommended. The application of ultrasound to a flooded canal

Figure 3 (A–F) Scanning electron microscopy of bacterial colonization with secondary apical periodontitis. Coronal and middle third portions: (A and C) amorphous materials and bacterial aggregates consisting of cocci and rods in the root canal lumen (×500, ×300, red arrows); (B and D) bacterial aggregates adhered to the root canal walls, colonized the dentinal surface, and progressed toward the dentinal tubules (×1000, ×1000); inset is ×5000 magnification. Apical third portion: (E) cocci and rods were present in the root canal lumen (×10,000, red arrows); (F) the root canal walls were heavily colonized with a dense bacterial biofilm consisting of cocci, rods, or filaments (×500); inset is ×5000 magnification.
goes further by warming the solution and creating violent turbulence to scrub canal walls and dentinal tubules. Ultrasoundically activated sodium hypochlorite is probably the most effective cleaning regime.

In addition, we mainly analyzed the invasion depth of bacteria in the middle and apical thirds of Group B and Group C because the invasion depth of the bacteria in the coronal area of each group was almost always <100 μm, and there were no bacteria in the middle and apical thirds of Group A. The invasion depths were deeper in the apical thirds of root canals with secondary apical periodontitis compared to primary apical periodontitis. However, no significant difference was observed in the middle thirds. The results further suggested that the complete eradication of bacteria is much more difficult to achieve in teeth with failed RCT than in teeth with primary apical periodontitis. Because chemomechanical procedures are unlikely to completely eradicate the root canal infection, the application of new and effective antimicrobial intracanal medication might be a valuable adjunct in the treatment of teeth with failed RCT.

In this study, we added a pulpitis experimental group, which has been mentioned only rarely in previous studies. The difference in bacterial distribution between teeth with pulpitis and chronic apical periodontitis was significant: teeth with pulpitis had bacteria mainly confined to the coronal third, failing to penetrate into the dentine tubules, whereas teeth with chronic apical periodontitis had bacteria distributed over the entire length of the root canal and penetrated into the dentine tubules. The difference clearly indicated that the removal of bacteria in teeth with apical periodontitis was much more difficult than in teeth with pulpitis. These results guided us toward targeted treatment for different types of pulpal and periapical disease. For pulpitis, we could obtain favorable outcomes after thorough crown-down preparation, which is less likely to be carried apically during subsequent preparation, reducing the incidence of blockage and postoperative flare-up. If time allows, RCT for pulpitis may be performed in one visit. There is a consensus that one-visit treatment is best to preserve an infection-free environment and periapical health. However, for maximum elimination of intracanal bacteria in the treatment of the teeth with apical periodontitis, we should combine more effective chemomechanical instrumentation with the application of disinfectants such as an interappointment dressing.

In summary, this descriptive study presented the complex bacterial colonization status of different types of infected root canals, which could explain why complete eradication is often unsuccessful. However, the quantity of the specimens used herein was somewhat limited. The specimens were required to conform to both the exact filter standards and extraction indications, which might have resulted in difficulty in taking samples and could have affected the experimental results. For that reason, we will attempt to overcome the limitation of sample acquisition in our future studies.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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References