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ORIGINAL ARTICLE

Prevalence of *Enterococcus faecalis* in saliva and filled root canals of teeth associated with apical periodontitis

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To investigate the prevalence of *Enterococcus faecalis* in saliva and filled root canals of patients requiring endodontic retreatment for apical periodontitis. Patients with apical periodontitis who were referred for endodontic retreatment were examined. The type and quality of the restoration, symptoms, quality of obturation were recorded. During retreatment, an oral rinse sample and root canal sample were cultured using brain-heart infusion agar and bile esculinazide agar to select for *E. faecalis*. The 16S rRNA technique was used to identify *E. faecalis.* A total of 32 women and 22 men (mean age: 38 years; s.d.: 11 years) and 58 teeth were studied. The prevalence of *E. faecalis* was 19% in the saliva and 38% in the root canals. The odds that root canals harbored *E. faecalis* were increased if the saliva habored this bacterium (odds ratio=9.7; 95% confidence interval=1.8–51.6; $P<0.05$). Teeth with unsatisfactory root obturation had more cultivable bacterial species in root canals than teeth with satisfactory root obturation ($P<0.05$). *E. faecalis* is more common in root canals of teeth with apical periodontitis than in saliva. The prevalence of *E. faecalis* in root canals is associated with the presence of *E. faecalis* in saliva.
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INTRODUCTION

Enterococci are common bacteria that inhabit the gastrointestinal tract, oral cavity, and vagina of humans and animals. Although enterococci were initially regarded as non-virulent, they are now recognized as one of the major causes of nosocomial infections worldwide.[1] In dentistry, *Enterococcus* species, in particular *Enterococcus faecalis*, have been found to be associated with chronic periodontitis[2] and failed root canal treatments involving chronic apical periodontitis.[3] Furthermore, in a study of 36 patients, *E. faecalis* was commonly isolated from periapical lesions refractory to endodontic treatment.[4]

A widely held assumption is that microorganisms found in the root canal space are derived from those colonizing the oral cavity.[5] However, it has recently become apparent that this hypothesis requires elaboration. Whereas *E. faecalis* was present in oral rinse samples from patients who had endodontic treatment,[5] this bacterium was rarely detected in healthy mouths.[6] Given the ubiquitous occurrence of enterococci in food products, Kampfer speculated that oral niches, such as untreated necrotic root canals, can become transiently colonized.[7] The microenvironment of root canals may especially favor the survival of enterococci and the establishment of long-standing local infections.[8] Other conditions, such as the quality of obturation, could conceivably also influence the colonization of *E. faecalis* and hence microflora in roots, either directly or indirectly.

Whether there were any differences between the presence of *E. faecalis* in saliva and in filled root canals is still unknown, and the difference between the presences in unsatisfactorily obturated canals and in well-obturated canals is also still unclear. In this study, we aimed to investigate the association between the prevalence of *E. faecalis* in saliva and in root canals of previously endodontically treated teeth that needed retreatment because of apical periodontitis. We also evaluated the association between the presence of *E. faecalis* in root canals and various clinical and physical variables, including the type and quality of restoration and the quality of obturation.

MATERIALS AND METHODS

Patient recruitment

Adult patients aged 18 years or older who attended the Peking University School and Hospital of Stomatology between September 2006 and December 2008 were invited to participate in this study if they showed radiological evidence of apical periodontitis based on the AAE accepted diagnostic recommendations[9] and also requiring endodontic retreatment. Patients who smoked, were pregnant, or had diabetes or other systemic conditions were excluded, as were those who had undergone treatment with local or systemic antimicrobial agents within the previous 6 months and those with generalized periodontal disease with a pocket depth of $\geqslant 4$ mm. Also excluded were patients requiring retreatment owing to missing canals, separated endodontic instruments, perforations or calcified root canals in which the apex was inaccessible. The study was approved by the Ethics Committee of Peking University Institutional Review Board. Patients were informed of the study protocol and aims, and written consent was obtained before recruitment.

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Clinical assessment

During the clinical examination and retreatment, the location of affected teeth, number of root canals per tooth, and clinical signs and symptoms (presence of pain, hypermobility, sinus tract and its origin, gingival or mucosal swelling and status of associated periodontal pockets of teeth needing retreatment) were recorded. The type (extracoronal or intracoronal) and quality (satisfactory or unsatisfactory) of final restorations were assessed according to criteria adapted from the 'Modified USPHS'.[10] Crowns and onlays, either alone or as a bridge abutment, were recorded as extracoronal restorations, whereas direct restorations of silver amalgam or composite resin and all indirect inlays were recorded as intracoronal restorations. Restorations were regarded as unsatisfactory if there were any secondary carious lesions, marginal defects that the explorer could penetrate, or any fracture or loss of the restoration. We assessed the quality of root canal obturation by examining periapical radiographs[11]. Obturation was considered unsatisfactory if (i) the end of the obturation filling was more than 2 mm short of the apex or had extruded beyond the apex; and (ii) space was visible laterally along the obturated canal or voids were present within the filling mass. Obturation was considered satisfactory if (i) there was uniform radiodensity and adaptation of the filling to the root canal walls; and (ii) the root filling ended short of the apex by 2 mm or less. All the clinical assessment was accomplished by two investigators together at the same time.

Bacterial sampling

Samples were collected from both saliva and root canals of each patient and cultured for bacteria. Saliva samples were collected by the method described by Sedgley.[6] Patients rinsed their mouths for 60 s with 10 mL of sterile distilled water and transferred the sample to a 50-mL polypropylene tube, which was stored at 4 ℃ and processed in the laboratory within 2 hours. Root canals were swabbed by using the method described by Gomes *et al.*[12]. The affected tooth was isolated with a rubber dam and disinfected with 5.25% sodium hypochlorite, which was inactivated with 5% sodium thiosulfate. Aseptic techniques were followed throughout endodontic therapy and sampling. When the previous restoration was removed and root canal orifice was loacted, the pulp chamber was disinfected with 5.25% sodium hypochlorite and the previous obturation was removed with Protaper nickel–titanium rotary instruments S1-F2 (Dentsply Tulsa Dental, Tulsa, OK, USA) under irrigation with sterile physiological saline solution. Canal patency was established with minimal instrumentation. Sterile saline was then used to wet the canal and a microbial sample was taken by inserting three sterile paper points into the full length of the canal and keeping them in place for 30 s. The debris located in the apical third of the root canal and the paper points were placed into a 2-mL centrifuge tube containing 1.5 mL viability medium Gotenberg agar III transport medium and samples were immediately assessed.

Laboratory assessment

Oral rinse samples were centrifuged at 4 ℃ for 10 min at 13 000*g* and pellets were resuspended in 1 mL of sterile nuclease-free water (Invitrogen, Carlsbad, CA, USA). Meanwhile, centrifuge tubes containing root-canal samples were shaken thoroughly in a mixer for 60 s, and log-10 serial dilutions were made with viability medium Gotenberg agar III transport medium. With the use of a spiral plater (Model D; Spiral Systems Inc, Cincinnati, OH, USA), a 50-μL inoculum of each prepared sample was plated onto brain-heart infusion agar plates and bile esculinazide agar plates to culture all bacteria and to select for enterococci, respectively. Plates were incubated aerobically for 48 h at 37 ℃. Colonies that were presumptively identified as enterococci based on bile esculinazide hydrolysis were purified by streak-plating onto a fresh esculinazide agar plate. Isolates were characterized as catalase-negative, non-motile, Gram-positive cocci if they were capable of growth in Todd Hewitt agar (Difco; Becton, Dickinson & Company, Sparks, NV, USA) supplemented with 6.5% sodium chloride at 42 ℃. They were then identified as *E. faecalis* with Analytical Profile Index 20 Strep identification kits (Bio Mérieux SA, Marcy-l'Etoile, France) and the16-S rRNA technique,[13] with type strain *E. faecalis* ATCC 29212 serving as a positive control.

To confirm the selective culture results, the *E. faecalis*-specific polymerase chain reaction (PCR) primer was prepared according to the method of Sedgley.[14] Universal bacterial primer positive control and *E. faecalis*-specific primer positive control were used in the procedure. The 25-μL reaction mixtures contained 0.5 μL of primer, 2.5 μL of 10×PCR buffer, 0.5 μL Taq DNA polymerase (Gibco BRL, Gaithersburg, MD, USA) and 0.5 μL deoxyribonucleoside triphosphates. The PCR protocol comprised an initial denaturation step at 95 ℃ for 15 min, followed by 35 cycles of a denaturation step at 94 ℃ for 20 s, a primer annealing step at 50 ℃ for 45 s, an extension step at 72 ℃ for 30 s and a final step of 72 ℃ for 5 min. Amplicons were analyzed by 1.5% agarose gel electrophoresis performed at 4 $V \cdot cm^{-1}$ in Tris-borate EDTA buffer. The gel was stained with 0.5 $mg \cdot mL^{-1}$ ethidium bromide and photographed under ultraviolet light (Figure 1).

Statistical analysis

The data were analyzed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). In the bivariate analysis, Fisher's exact test was used to assess the statistical significance of the association between the presence of *E. faecalis* in root canals and its presence in saliva, tooth location (anterior or posterior), number of root canals (single or multiple),

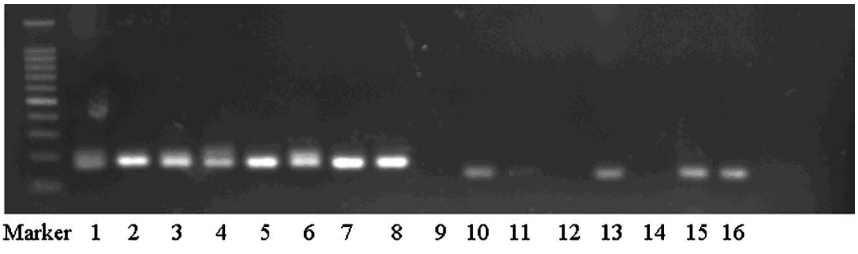

Figure 1 Identification with 16S-rRNA technique. Lane 1, universal bacterial primer negative control; lane 2, universal bacterial primer positive control; lanes 3–8, transcription amplification of microbial samples; lane 9, *E. faecalis* specific primer negative control; lane 10, *E. faecalis* specific primer positive control; lanes 11–16, transcription amplification of microbial samples.

Table 1 Presence of *E. faecalis* in root canal, by selected characteristics

Characteristics		*E. faecalis* in root canals: Yes	No	Total	*P* value
Age/year, mean (s.d.)		41 (12)	36 (10)	38 (11)	0.102
No. of roots per tooth, mean (s.d.)		1.3 (0.6)	1.4 (0.7)	1.3 (0.7)	0.813
Sex	Male	9	13	22	0.775
	Female	11	21	32	
Salivary *E. faecalis*	Yes	8	2	10	0.004
	No	14	30	44	
Tooth location	Anterior	11	22	33	0.411
	Posterior	11	14	25	
Clinical signs and symptoms[a]	Yes	3	3	6	0.664
	No	19	33	52	
Coronal restorations	Extracoronal	5	17	22	0.732
	Intracoronal	6	30	36	
Restoration quality	Satisfactory	7	11	18	1.000
	Unsatisfactory	15	25	40	
Canal obturation quality	Satisfactory	3	8	11	0.507
	Unsatisfactory	19	28	47	

[a] Signs and symptoms included presence of pain, hypermobility, sinus tract and its origin, gingival and mucosal swelling, and associated periodontal pockets.

presence of clinical signs and symptoms which were mentioned above (yes or no), type of coronal restoration (extracoronal or intracoronal), restoration quality (satisfactory or unsatisfactory) and quality of canal obturation (satisfactory or unsatisfactory). The Chi-square test was performed if at least 80% of cells had an expected frequency of at least 5 and no cell had an expected frequency of less than 1. The *t*-test was used to analyze whether age and number of roots differed according to the presence or absence of *E. faecalis* in root canals, and whether the number of bacterial species cultured from the root canal differed according to the obturation quality. The data will be assessed for normal distribution properties using the Shapiro–Wilk test for normality. Logistic regression analysis was conducted to assess the effects of the above independent variables in a multivariate model in which the presence of *E. faecalis* in root canals (1=yes; 2=no) was the independent variable. The cutoff point for statistical significance was set at 0.05.

RESULTS

Fifty-four patients, 32 men and 22 women, who required endodontic retreatment, were recruited in this study. Their ages ranged from 18 to 70 years and their mean age was 38 years (s.d.: 11 years). A total of 58 teeth—29 incisors, 4 canines, 19 premolars, and 6 molars—were studied. In the present study, there were 6.8% of teeth (4/58) with negative culture on the plates.

The prevalence of *E. faecalis* was 19% (10 in 54 patients) in saliva and 38% (22 in 58 teeth) in root canals. A statistically significant association ($P<0.05$) was found between the presence of *E. faecalis* in saliva (10/54) and its presence in root canals (22/58) (Table 1). Only the presence of *E. faecalis* in saliva remained significantly associated with increased odds of identifying *E. faecalis* in root canals (odds ratio=9.7; 95% confidence interval=1.8–51.6; $P<0.05$) (Table 2). The Cox and Snell R^2 value was 0.144.

The tooth locations, presence of clinical symptoms, type of coronal restoration, restoration status, number of root canals and quality of canal obturation were not significantly associated with the presence of *E. faecalis* in root canals. These findings were confirmed in the logistic regression analysis, so these variables were removed from the final model.

Using our culture method, we found up to four identifiable species of bacteria in their roots (Table 3). The overall mean number of bacterial species identified was 2.3 (s.d.: 1.1) (Table 4). All enterococcal isolates were identified as *E. faecalis*. We found that 17 teeth had a single identifiable species of bacteria in their roots. Of these 17 teeth, 5 harbored *E. faecalis*. Among the teeth that required retreatment, those showing satisfactory canal obturation were more likely to harbor only a single cultivable species of bacteria ($P<0.05$) and had a lower mean number of cultivable bacterial species ($P<0.05$) than those showing unsatisfactory obturation.

Table 2 Final logistic regression model of *E. faecalis* in root canals

Factors	β (s.e.)	Odds ratio (95% CI)	*P* value
Presence of *E. faecalis* in saliva			
Yes	−2.274 (0.852)	9.7 (1.8–51.6)	0.003
No[a]			
Constant	0.887 (0.318)		0.005

[a] Reference category.

Table 3 No. of identifiable species of bacteria and the presence of *E. faecalis* in the root canals

E. faecalis	No. of identifiable species of bacteria: 1	2	3	4	Total
Yes	5	6	6	3	20 (37%)
No	12	4	14	4	34 (63%)
Total	17	10	20	7	54 (100%)

Table 4 Bacterial profile in non-selective medium according to quality of root canal obturation

Bacteria identified	Root canal obturation: Satisfactory	Unsatisfactory	All	*P* value
Mean no. (s.d.)	1.1 (0.3)	2.6 (1.0)	2.3 (1.1)	<0.05
% teeth with only one species	89%	20%	32%	<0.05

DISCUSSION

In the present study, *E. faecalis* could be found in root canals of teeth with apical periodontitis requiring endodontic retreatment or in the saliva, or in both. The prevalence of *E. faecalis* in root canals and saliva was 38% and 19%, respectively. These results agree with those of other studies. For example, the reported prevalence of *E. faecalis* in root canals ranges from 32% to 70%, with *E. faecalis* being the predominant species in filled root canals with persistent apical periodontitis.[12,15–17] The reported prevalence of *E. faecalis* in oral saliva of endodontic patients is lower, at 10%–17%.[5–6] In contrast, an early study reported that 75% of endodontic patients had detectable *Enterococcus* species in saliva.[18] The conflicting results indicates more clinical studies should be performed to test the hypothesis that oral cavity provides an available source of *E. faecalis* to enter root canals.

Although *E. faecalis* was detected less frequently in oral rinse (10%) than from tongue (42%) and gingival sulcus (14%),[5] it is more likely that *E. faecalis* from oral rinse stays in a planktonic situation, and gets more chance to enter the root canal system than that from tongue or gingival sulcus with a biomembrane structure. We focused on the planktonic *E. faecalis* in oral cavity in this study.

The most obvious source of *E. faecalis* in failed root canals is thought to be the oral cavity. However, our findings suggest the prevalence of *E. faecalis* in saliva of patients undergoing endodontic retreatment is low. Since most root canal samples that were positive for *E. faecalis* corresponded to saliva that were also positive, there could an association between the presence of *E. faecalis* in root canals and its presence in saliva. We did not include healthy controls for the saliva tests, but the literature generally suggests that *E. faecalis* is also not a common oral colonizer in oral cavities with healthy dentition. No detectable enterococci were found in the saliva of dentate people who had never received endodontic treatment.[6,8]

It should be noted that cross-sectional studies are limited, being single views of a microflora existing in a complex dynamic environment and not reproducible within the same individual on different occasions. Future studies could examine the prevalence and characteristics of enterococci recovered in longitudinal studies. If a transient oral infection with *E. faecalis* occurred during or after treatment, the bacteria might enter the unsealed root canal. If the conditions of root canals favor the survival of enterococci, a long-standing local infection may become established.[8] Still, the oral cavity seems to be the most likely source of *E. faecalis* in failed root canal treatment. A laboratory study showed that *E. faecalis* in the starvation phase could develop a biofilm on human dentin. *E. faecalis* in a biofilm has much more resistance to 5.25% sodium hypochorite than stationary cells, and resistance increased as the biofilm matured. Such a mechanism may contribute to the predominant role of *E. faecalis* in persistent periapical infections,[19] but unanswered questions include when and how the bacteria enter filled root canals, and what variables in primary endodontic treatment may make the root canal microenvironment conducive to bacterial growth.

The quality of obturation often reflects the quality of endodontic treatment; in addition, roots which are clean and of proper shape are generally easy to obturate satisfactorily. In this study, the microflora of unsatisfactorily obturated root canals was mixed, whereas that of satisfactorily obturated root canals harbored only one species based on the culture technique, probably owing to the lack of space or nutrients to support more than one type of microorganism. In addition, the culture-based technique would impact the biodiversity found which was mainly discussed in the latter part. Our findings agree with those of other researchers. For example, Gomes found that obturated teeth generally harbored one to two species, with facultative anaerobic and Gram-positive bacteria predominating.[12] Sundqvist reported a similar result and suggested that the number of species isolated from retreatment cases was probably contingent on the quality of the initial endodontic treatment.[20] The results of this study also agree with Sundqvist that teeth with inadequate endodontic treatment are more likely than teeth with apparently well-cleaned canals to have a microflora similar to that found in untreated canals, and also more likely to contain a greater number of species. Studies reported that the increased occurrence of Gram-positive bacteria in particular may be a consequence of their strong resistance to instrumentation and antiseptic agents.[13,20]

We found that *E. faecalis* was more common in root canals showing unsatisfactory obturation than in those showing satisfactory obturation (19/58 *vs.* 3/58), but the prevalence was not significantly associated with the quality of obturation. Unsatisfactorily obturated root canals could provide more space and nutrition than well-obturated canals, and the available space may create a facultative anaerobic environment. In contrast, well-obturated canals maintain an obligate anaerobic environment that does not favor the survival and growth of *E. faecalis*. Inadequate cleaning and shaping may also have left infected debris behind. Microorganisms such as *E. faecalis* can survive within the small canals of apical ramifications or in the space between the root filling and canal wall. In fact, *E. faecalis* strains can survive for at least 6–12 months in an environment where nutrients are scant and when commensality with other bacteria is reduced.[6–7] *E. faecalis* is also extremely resistant to chemicals, including calcium hydroxide.[21–23]

E. faecalis has been commonly located in the apical third of the root canal, suggesting that invasion might have occurred during endodontic treatment.[24] It has also been suggested that a positive association may exist between the occurrence of *E. faecalis* and the number of clinic visits, owing to coronal microleakage through the temporary filling placed between endodontic treatment sessions.[25] During the coronal restoration phase, obturated root canals may become exposed to the oral cavity at some point during treatment, especially when temporary restoration is required during the construction of an indirect restoration. Thus, the quality of the temporary restoration may be important to the prevalence of *E. faecalis* infection in root canals.[26]

A study found *E. faecalis* was resistant to removal by root canal preparation followed by intracanal dressing.[27] *E. faecalis* has been found more frequently in filled canals without a radiographic lesion than in those with a lesion, suggesting that bacterial entry can happen after obturation.[9] On the other hand, extracoronal restoration with a satisfactory margin can reduce the risk of post-treatment extracoronal leakage and increase the success rate of root canal therapy.[28] In this study, however, we did not find any association between restoration quality or type and prevalence of *E. faecalis* in root canals or saliva. This result could be due to the low sensitivity of using Fisher's exact test on small samples.

It should be noted that our culture technique is commonly used for assessing *E. faecalis* in saliva.[29] Furthermore, to confirm the identity of *E. faecalis*, we included a molecular method of PCR. Researchers using molecular methods have recently demonstrated that the microflora associated with endodontic infections is more diverse than that reported by researchers using only conventional culture methods and PCR was more sensitive than culturing in the detection of *E. faecalis*.[30] But the culture and Analytical Profile Index methods could be used to detect the activity of the isolated *E. faecalis*. Combining molecular and culture technique is probably the best approach available to provide comprehensive information about the microflora

associated with endodontic infections. As an illustration of this point, *E. faecalis* had been isolated in fissure caries using a conventional culture technique, at three orders of magnitude below counts of *Streptococcus mutans*.[31] With the use of modern culture and molecular identification techniques, however, enterococci could not be found in carious dentin.[32] Thus, it seems unlikely that enterococci could occur in carious lesions and act as a source of *E. faecalis* in root canals.

In the present study, there were 6.8% of teeth (4/58) with negative culture on the plates. Earlier studies were unable to isolate bacteria from 20%,[16] 15%[15] and 10%[12] of teeth. However, failure to detect bacteria does not prove their absence; it is possible that some microorganisms could have been lost, especially if the number of microorganisms present in the root canal was very low or microorganisms were the obligate anaerobes.[15] This information should need further studies to figure out.

In conclusion, we have shown here that the prevalence of *E. faecalis* in root canals is associated with the presence of *E. faecalis* in saliva. Besides thorough root canal cleaning, shapings and obturation, maintaining hermetic coronal sealing during and after root canal treatment is essential to prevent *E. faecalis* contamination and colonization of root canals. It is clear that further, larger studies are needed to definitively support or challenge the theory that enterococci can enter the root canal system during or after root canal treatment.

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