Investigation of the effect of the coronal restoration quality on the composition of the root canal microflora in teeth with apical periodontitis by means of T-RFLP analysis

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Abstract

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Aim To investigate the effect of the radiographic and clinical quality of coronal restorations on the composition of the root canal flora of teeth with necrotic pulps and teeth with root fillings associated with apical periodontitis.

Methodology Twenty-eight necrotic pulps and 35 root filled canals with signs of apical periodontitis were studied. Both the coronal filling (presence of radiographically or clinically deficient margins and/or secondary caries) and the root filling (homogeneity and length) were scored. Bacterial root canal samples were taken with sterile paper points under rubber dam and using measures to prevent contamination. A DNA-based nonculture bacterial identification technique was used, namely terminal restriction fragment length polymorphism (T-RFLP) analysis.

Results Twelve samples were negative for bacterial DNA. A total of 33 different terminal restriction fragments (TRFs) were detected. The *Fusobacterium nucleatum*/*Streptococcus mitis* group was the most frequently encountered TRF. The mean number of TRFs per necrotic pulp was 6.2 and 5.8 for the groups with acceptable and unacceptable coronal restorations, respectively. This difference was not significant. In the root filled group, these values (respectively, 5.2 and 8.6) were statistically significantly different ($P < 0.05$). The following parameters in root filled teeth had no significant influence on the mean numbers of TRFs detected: the length and homogeneity of the root filling and the type of tooth (anterior–premolar–molar).

Conclusion T-RFLP allowed the rapid assessment of bacterial biodiversity in root canal samples. The technique revealed the presence of bacteria that have rarely been described in the root canals of teeth with apical periodontitis. Biodiversity in the root filled group was high, as compared with culture-dependent studies where monoinfections were more frequently reported. Only in root filled teeth did defective coronal restorations have a statistically significant influence on the mean numbers of detected TRFs per sample.

Keywords: apical periodontitis, bacterial analysis, endodontics, necrosis, root filled teeth, T-RFLP.

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Introduction

Coronal leakage has been recognized as a cause of failure in root canal treatment (Saunders & Saunders 1994). A number of *in vitro* studies have demonstrated the possibility of bacterial leakage along root fillings as well as along temporary coronal restorations (Khayat *et al.* 1993, Deveaux *et al.* 1999). These findings were

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supplemented by data from *in vivo* studies that indirectly described the effect of coronal leakage (Ray & Trope 1995, Tronstad *et al.* 2000, Hommez *et al.* 2002). These studies gave evidence of an increased incidence of apical periodontitis associated with defective root fillings and with leaking coronal restorations.

Bacteria are present in the oral cavity and potentially leak into root canals exposed to the oral environment. The analysis of these complex bacterial communities has classically been restricted to conventional culture methods. These methods are time-consuming and laborious. Furthermore, approximately 50% of the oral microflora cannot be cultured (Socransky *et al.* 1963). Others have shown that the serial dilution anaerobic culture procedure recovers only 20–70% of the microscopic count (Mombelli *et al.* 1989). Culture-independent molecular techniques, frequently based on the detection of 16S rRNA genes, have been developed with the aim of including nonviable, uncultivable and fastidious bacteria. These techniques include fluorescent *in situ* hybridization (FISH) (Langendijk *et al.* 1995), denaturating gradient gel electrophoresis (DGGE) (Heuer & Smalla 1997), temperature gradient gel electrophoresis (TGGE) (Heuer & Smalla 1997), temporal temperature gradient gel electrophoresis (TTGE) (Vasquez *et al.* 2001) and cloning (Suau *et al.* 1999, Munson *et al.* 2002). This study used terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu *et al.* 1997) to describe the microflora in root canals with necrotic pulps, as well as in root fillings associated with post-treatment disease. Thus far, only one study used T-RFLP for the characterization of the oral bacterial microflora in the saliva of healthy subjects and of patients with periodontitis (Sakamoto *et al.* 2003). Techniques such as T-RFLP may have the additional advantage of high throughput and reproducibility for monitoring bacterial communities (Osborn *et al.* 2000, Blackwood *et al.* 2003), thus providing a rapid method of finding major differences between communities and testing hypotheses based on a comparison of samples (Blackwood *et al.* 2003).

Culture-based information is available on the composition of the bacterial microflora in necrotic root canals, and in root canal fillings associated with failure (Dahlén & Haapasalo 1998). Molecular-based techniques were also used to investigate the microflora of root canals with apical periodontitis (Conrads *et al.* 1997, Rolph *et al.* 2001, Munson *et al.* 2002, Siqueira *et al.* 2002). However, the effect of defective coronal restorations on a possible shift in the composition of the root canal microflora in teeth with apical periodontitis has not yet been investigated.

The aim of the present study was therefore to investigate whether it was possible using T-RFLP to identify the effect of the radiographic and clinical quality of the coronal restorations on the composition of the root canal microflora in teeth with necrotic pulps and teeth with root fillings associated with apical periodontitis.

Materials and methods

Case selection

The 63 patients selected in this study all received conventional root canal treatment or retreatment. The patients were healthy adults (20 males and 43 females), with an average age of 40.3 ± 14.0 years. This study was approved by the Ethics Committee of the Ghent University Hospital (No. 2003/006). Informed consent was obtained from all participants.

The samples were taken from previously untreated root canals with necrotic pulps ($n = 28$) or filled root canals (retreatment cases) ($n = 35$). All teeth included in the study had radiographic signs of apical periodontitis visible on a pretreatment radiograph (i.e. at least twice the width of the normal periodontal ligament).

Data on coronal restorations were recorded. A coronal restoration was categorized as deficient when one of the following criteria was present: radiographic signs of deficient margins on the pretreatment radiograph, clinical signs of a physical defect detected with a probe penetrating the tooth-restoration interface, secondary caries, partial or total loss of restoration. These parameters coincide with the criteria used in a previous study on coronal leakage (Hommez *et al.* 2002).

The following cases were excluded from the study:

- Teeth with a periodontal pocket extending to the apical third of the tooth.
- Teeth that could not be suitably isolated from the gingiva and saliva.
- Patients who received antibiotic treatment within the preceding 3 months.
- Patients with systemic disease.

The quality of an existing root filling was assessed on a pretreatment radiograph according to length and homogeneity. The length was categorized as acceptable when the root filling terminated 0–2 mm from the root apex. Overextension and underextension of the root filling was scored when the apical extent of the root filling ended, respectively, beyond the root apex and more

than 2 mm from the root apex. A root filling was scored homogenous when no voids were present and the root filling was well compacted in the root canal along the whole length of the root filling.

Sampling procedure

The teeth selected for bacterial sampling were first cleaned with pumice and a rubber dam was placed. Defective coronal fillings and caries, if present, were removed and the teeth and rubber dam were subsequently disinfected with 30% hydrogen peroxide and a 10% iodine tincture, according to the protocol proposed by Möller (1966). After the tincture had dried, the tooth surface was swabbed with a 5% sodium thiosulphate solution to inactivate the iodine tincture so that remnants of iodine would not influence the bacteriological sample.

Subsequently, canal access was gained and enlarged with Gates Glidden drills (Dentsply Maillefer, Ballaigues, Switzerland). If present, the coronal part of the root filling was removed with Gates Glidden drills and the more apical part of the root filling was partially removed using K-Flexofiles (Dentsply Maillefer). Instrumentation was carried out as close as possible to 2 mm from the apical constriction. This was checked with an electronic apex locator (Apex Finder AFA; SybronEndo, Orange, CA, USA). A small amount of saline (0.85%) was introduced in the canal with a sterile syringe. The fluid was then mixed with the contents of the root canal with a root canal file, making pumping movements. The canal fluid was subsequently soaked into a sterile paper point. The paper points were directly transferred to a tube containing sterile physiological water. The transport tubes were transferred to the laboratory within 30 min. The root canal instrumentation was continued after the sampling procedure.

DNA extraction

For DNA extraction of bacteria that were collected on paper points, the QIAamp DNA mini kit (Qiagen, Hilden, Germany) was used according to the manufacturer's recommendations, with minor modifications. After 10-min centrifugation at 5000 ***g***, the pellet was resuspended in 180 μL of lysis buffer (20 mmol L^{-1} Tris–HCl, pH 8.0; 2 mmol L^{-1} EDTA; 1.2% Triton). Fifty units of mutanolysin (25 U μL^{-1}) (Sigma, Bornem, Belgium) were added and the samples were incubated for 30 min at 37 °C. After the addition of 20 μL Proteinase K (20 mg mL^{-1}) and 200 μL AL buffer (Qiagen), samples were incubated for 30 min at 56 °C. Next, 200 μL of ethanol was added and DNA was purified by adding the lysate to the Qiagen columns as described by the manufacturer. Finally, the total bacterial DNA was eluted with 100 μL of AE buffer. DNA extracts were stored at −20 °C.

T-RFLP analysis

The forward primer 10f (5′-AGTTTGATCCTGGCTCAG) and the reverse primer 534r (5′-ATTACCGCGG-CTGCTGG) (Muyzer *et al.* 1993), which target the 16S rRNA gene (16S rDNA) of the domain *Bacteria*, were used to amplify part of the 16S rDNA by PCR. A 15-μL PCR mixture contained 0.1 μmol L^{-1} of each primer, 7.5 μL of Promega master mix (Promega, Madison, WI, USA), 1.5 μL of sample and distilled water. Thermal cycling consisted of an initial denaturation of 5 min at 94 °C, followed by three cycles of 1 min at 94 °C, 2 min at 50 °C and 1 min at 72 °C, followed by 35 cycles of 20 s at 94 °C, 1 min at 50 °C and 1 min 72 °C, with a final extension of 10 min at 72 °C, and cooling to 10 °C. A 20-μL restriction mixture, containing 1 μL PCR product, 1 μL of *Bst*UI (Westburg, Leiden, the Netherlands) and 4 μL of the appropriate buffer, was incubated at 60 °C for 3 h. Ten microlitre of the restriction reaction was purified by ethanol precipitation. The obtained pellet was solved in 13.1 μL deionized formamide (AMRESCO, Solon, OH, USA), 0.1 μL ROX500 and 0.3 μL HD400 GeneScan size standards (Applied Biosystems, Foster City, CA, USA) followed by denaturation at 96 °C for 2 min and immediate cooling on ice. The fluorescently labelled terminal restriction fragments (TRFs) were electrophoresed on an ABI PRISM 310 (Applied Biosystems). Figure 1 shows the output of a T-RFLP profile. TRFs with a peak height less than 10% of the highest peak were excluded from the analysis, as cloning studies (unpublished data) indicated that such peaks did not correspond with any of the species shown to be present by cloning.

Construction of the T-RFLP library

The T-RFLP pattern obtained from a mixed sample consists of the 5′ terminal *Bst*UI restriction fragments obtained from amplified rDNA of the different species present. Theoretically the number of peaks (TRFs) reflects the number of different species present in a sample. Identification of the peaks in a T-RFLP pattern, in other words assignation of a species name to each

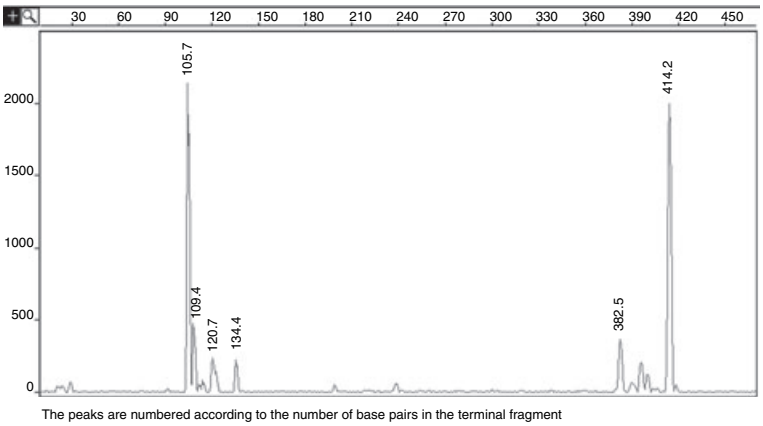

Figure 1 Terminal fragment length polymorphism analysis (T-RFLP) of a necrotic root canal with leakage.

TRF, is based on comparison with a library composed of the T-RFLP patterns of well-identified species. Such library T-RFLP patterns consist of a single TRF, as they are obtained from pure cultures of a single species. Another means to obtain T-RFLP patterns of single species is by carrying out computer-assisted (i.e. virtual) restriction analysis of published 16S rRNA sequences or by carrying out restriction analysis of cloned 16S rDNA, which is also sequenced to obtain the species name.

Data analysis

T-RFLP patterns were obtained as table files from the Genescan Analysis software and used in BaseHopper, a software program developed at our university (Baele *et al.* 2000). Using these sample files containing TRFs (peak values) in base pairs, this program enabled us to construct manually a library which contains one entry for each species and whereby each entry consists of a numeric value representing the peak value in base pairs. The peak values in the library entries are the averages of the peak values obtained after testing different strains or cloned 16S rRNA genes of each species.

Data analysis and statistical analysis were performed in SPSS 11.0.1 (SPSS Inc., Chicago, IL, USA).

The mean number of TRFs per sample in the different groups were analysed with the Student's *t*-test or ANOVA when more than two groups were present.

Results

A total of 12 samples, of which nine in the root filled group, were PCR-negative. Of the 51 PCR-positive samples, 19 teeth showed signs of defective coronal restorations. Both the pulp necrosis group and the root filled group were divided into subgroups of teeth with acceptable and unacceptable coronal restorations. Table 1 gives an overview of the TRFs and the corresponding species or species groups present in the different patient subgroups. A total of 33 different TRFs were detected. The number of TRFs detected per sample ranged from 0 to 12 for the pulp necrosis group and 0 to 14 for the root filled group. Thirteen TRFs were present in all four groups. Among these, the TRF with a length of 25 bp (briefly TRF25), designated as the *Atopobium–Treponema* group, TRF105 (*Fusobacterium nucleatum*/*Streptococcus mitis* group), TRF109–110 (an unidentified *Veillonella* sp.), TRF111 (*Prevotella* spp.), TRF120 (the *Cartonella*/*Peptostreptococcus*/*Eubacterium* group) and TRF410 (the *Campylobacter curvus*/*Dialister* sp. group) were present in 19 or more of the patients. All 33 TRFs, except TRF452 (*Prevotella heparinolytica*), were present in the root filled group. Only three TRFs: TRF241 [the *Actinomyces israelii*/*Enterococcus faecalis* group), TRF245 (*Actinomyces* sp./*Lactobacillus casei* group/*L. crispatus*) and TRF213 (unidentified isolate ED06B)] were not present in the necrosis group. TRF105 (representing the *Fusobacterium nucleatum*/*Streptococcus mitis* group) was the most frequently encountered TRF: (57.7% of the samples), followed by TRF109–110 (*Veillonella* sp.) in 28 samples (53.5%). TRF109–110 was the most frequent species (15 of 25 samples) in the pulp necrosis group. In the root filled group, TRF105 (*Fusobacterium nucleatum*/*Streptococcus mitis* group) was counted 17 times (out of 27). The mean number of TRFs (i.e. species) per sample for the necrosis group was 6.21 and 5.82 for the sound coronal and defective coronal restoration group,

Table 1 Results of the T-RFLP analysis in relation to the tooth subgroups ($n = 63$)

		Groups				
		Necrosis		Filled (retreatment)		
	TRF length (bp)	No leakage	Leakage	No leakage	Leakage	Total
Number		17	11	27	8	63
Negative		3	0	6	3	12
Species						
Atopobium parvulum/ *Cryptobacterium curtum*/ *Olsenella* genome sp. C1/ *Peptostreptococcus lacrimalis*/ *Slackia exigua*/ *Treponema* spp.	25	7	4	10	3	24
Actinomyces sp./*Lactobacillus* spp.	52–53		1	1	1	3
Capnocytophaga sp.	95	1	1	1		3
Capnocytophaga gingivalis/*C. sputigena*	99	1		1		2
Capnocytophaga ochracea	102	1		2		3
Fusobacterium nucleatum/ *Streptococcus mitis* group	105	7	6	14	3	30
Veilonella sp.	109–110	11	4	10	3	28
Prevotella sp.	111	6	7	3	3	19
Prevotella sp.	114	4	4		2	10
S. gordonii/*Streptococcus milleri* group	115	2	2	5		9
Catonella morbi/ *Peptostreptococcus magnus*/ *P. octavius*/*Eubacterium saphenum*/ *E. tardum*	120	8	4	11	3	26
Peptostreptococcus sp. oral clone CK035	135	2	3	2	2	9
Unidentified clone ED52c4	200	1	1	3	1	6
Unidentified isolate ED006B	213				1	1
Propionibacterium granulosum/ *Propionibacterium* sp./ unidentified oral bacterium ED52-1	220	3		2	2	7
Propionibacterium spp.	222		2	2		4
Propionibacterium acnes/*P. freudenreichii*	225	1		1	1	3
Peptostreptococcus sp. oral clone/*Staphylococcus* sp.	231	1		1		2
Peptostreptococcus micros	238	7	4	2	2	15
Actinomyces israelii/*Enterococcus faecalis*	241			5	1	6
Actinomyces sp./*Lactobacillus casei* group/*L. crispatus*	245			5	1	6
Campylobacter (Bacteroides) gracilis/*C. showae*	271	1		2	1	4
Lactobacillus catenaformis	383	3	3	2	1	9
Pseudomonas stutzeri/*Enterobacteriaceae*	385–387	2	1		1	4
Citrobacter freundii/ unidentified oral bacterium ED25E/ *Neisseria elongata*/ *Solobacterium moorei*	390	1	2	9	4	16
Rothia dentocariosa	393	1		1	1	3
Staphylococcus hominis	396	3	2		1	6
Selenomonas sputigena	399	1	1	1		3
Selemonas sp oral clone ED52c21	405	4	2	6	1	13
Campylobacter curvus	410	5	5	6	3	19
Dialister pneumosintes	414	3	1		1	5
Lactobacillus gasseri	417		2	1		3
Prevotella heparinolytica	452		2			2
Peaks not assigned to any TRF (17 different lengths)	A					43
Total number of TRFs detected		87	64	109	43	346
Number of different TRFs detected		26	23	27	24	
Mean number of TRFs per sample (negative samples not included)		6.21	5.82	5.19	8.60	

respectively. This difference was not statistically significant. In the root filling group, the mean number of TRFs was, respectively, 5.19 and 8.60. This difference was statistically significant ($P < 0.05$).

Table 2 presents the number of TRFs in filled root canals according to the quality of the root filling and the tooth type. None of the parameters scored had a significant influence on the number of TRFs detected per sample.

Three TRFs (TRF200, 213 and 405) could be linked to a clone or an isolate, but 16S rRNA gene sequencing of both clone and isolate yielded unidentified organisms. Furthermore, 17 different TRFs, observed in a total number of 43 cases, could not be assigned to any organism. For example, TRF404 was observed in four samples, but no clone or organism could be associated with it.

Discussion

Dental periapical diseases are caused primarily by the spread of microorganisms and bacterial elements (toxins and endotoxins) from the root canal into the periapical area. Apical periodontitis is a polymicrobial infection dominated by obligately anaerobic bacteria (Dahlén & Haapasalo 1998). The number of different species per case has been found to be relatively small, normally between two and eight, and with never more than 20 species in one root canal (Dahlén & Haapasalo 1998).

This study evaluated the usefulness of a molecular technique, namely T-RFLP for the study of dental periapical diseases.

Table 2 The number of TRFs in retreated teeth according to the quality of the root filling and the tooth type ($n = 26$)

		TRFs per sample	
	n	Mean	SD
Length of the root filling			
Acceptable	7	5.9	2.80
Short	15	6.0	3.5
Overextended	1	7.0	–
Unknown	3	4.7	0.6
Homogeneity of the root filling			
Homogeneous	7	6.6	2.5
Inhomogeneous	16	5.8	3.5
Unknown	3	4.7	0.6
Tooth type			
Front	6	5.3	1.4
Premolar	6	5.7	3.3
Molar	14	6.1	3.5

Negative samples were not included in this calculation.

The choice of the restriction enzyme used is important. *Bst*UI was chosen, based on *in silico* analysis of 16S rRNA genes and on the results of previous research (Engebretson & Moyer 2003), indicating that this restriction enzyme was well suited for maximal differentiation between bacterial species based on the length of the terminal 5′ restriction fragment of their 16S rDNA, i.e. their TRF. Nevertheless, several species turned out to have the same TRF. For example, after restriction with *Bst*UI, the species *Atopobium parvulum*, *Cryptobacterium curtum*, *Olsenella* genomospecies C1, *Peptostreptococcus lacrimalis*, *Slackia exigua* and *Treponema* sp. were shown to have a TRF with a length of 25 bp. This means that the occurrence of this TRF in a T-RFLP pattern does not allow differentiation between these different species.

A total of 33 different TRFs were detected in the present study. The number of TRFs per sample ranged from 0 to 12 in necrotic pulps and 0 to 14 in filled root canals. Although the average number of TRFs per canal that was detected in filled root canals (5.63) was lower than that in necrotic pulps (6.04), this difference was not statistically significant. These results are in opposition to those studies using culture methods where it was shown that the microflora in filled root canals exists mostly as monoinfections (Molander *et al.* 1998, Sundqvist *et al.* 1998). However, in cases with poor quality root fillings, the composition of the microflora resembled that of untreated root canals. This difference is assumed to be a consequence of inadequate cleaning (Sundqvist *et al.* 1998, Cheung & Ho 2001). In this study no evidence of this assumption was seen, as the length and homogeneity of the root filling had no statistically significant influence on the number of TRFs detected in each sample.

Enterococci are found in approximately one-third of the filled root canals with apical periodontitis (Möller 1966, Fraser 1974, Molander *et al.* 1998). *Enterococcus faecalis* appears to be extremely resistant to the antibacterial agents used during root canal treatment and it is one of the few microorganisms that can survive the anti-bacterial effects of dressing with calcium hydroxide (Byström *et al.* 1985). Enterococci have also been shown to have the ability to survive in root canals as single organisms without the support of other bacteria (Fabricius *et al.* 1982). The TRF corresponding to *E. faecalis* or *A. israelii* (TRF241) was only present in the root filled group. Both bacteria were isolated, although the former more frequently, from filled root canals by other authors (Molander *et al.* 1998, Sundqvist *et al.* 1998, Hancock *et al.* 2001).

On the contrary, Rolph *et al.* (2001), using molecular methods, failed to detect both. There is no obvious explanation for this difference in incidence of isolation of *E. faecalis* between the studies.

No information was available regarding the (possibly leaking) temporary fillings or the number of visits spent on the original root canal, which can also influence the presence of *E. faecalis* (Siren *et al.* 1997). The presence of defective coronal restorations did not seem to have effect on the presence of TRF241. In teeth with necrotic pulps, TRF 241 was absent. The incidence of TRF 241 was about the same for the samples taken from root filled teeth with unacceptable and acceptable coronal restorations (18.5 and 12.5% of the samples, respectively).

No study has yet investigated the effect of the radiographic and clinical quality of the coronal restorations on the composition of the root canal microflora in teeth with necrotic pulps and teeth with post-treatment disease *in vivo*. No specific species or group of species was found that indicated leakage. It was interesting to find that in root filled teeth with defective coronal restorations, the mean number of TRFs per sample detected was significantly higher than in other cases. Although care should be taken when interpreting this result because of different sample sizes, this difference in the mean number of TRFs per sample from teeth with unacceptable and acceptable coronal restorations could be explained by ingress of bacteria that increased the diversity of the microflora. Nevertheless, no similar significant difference could be detected in the samples from necrotic pulps.

Care should be taken when defining defective restorations (restorations that were scored radiographically and/or clinically unacceptable in this study) as restorations with coronal leakage. A minute gap at the coronal or radicular-restoration interface, which would not be visible on the radiograph, could be sufficient to permit bacterial penetration.

Several explanations are possible for the fact that 12 samples produced negative results with the T-RFLP technique. As the majority of these negative samples were found in the root filled group, it is not inconceivable that some of the cases were healing and that a restricted amount or no bacteria were present in the root canal. Apical radiolucencies are not necessarily caused by intracanal infection. A radiolucency may refer to a periapical cyst (Nair *et al.* 1996), a foreign body reaction (Nair *et al.* 1990, Sjögren *et al.* 1995) or scar tissue (Bhaskar 1966). It may also have been possible that errors in sampling resulted in negative samples. It was also possible that microorganisms were present but that the sampling procedure did not pick-up the bacteria in some parts of the root canal. It should be noted that more negative samples were found with culture-based methods (Molander *et al.* 1998: 32.0%, Cheung & Ho 2001: 33.3%, Sundqvist *et al.* 1998: 55.6%) than with the DNA technique used in the present study (19.0%) and in other studies (Conrads *et al.* 1997: 17.6%, Siqueira *et al.* 2002: 0%), although Rolph *et al.* (2001), who also used T-RFLP as well, reported 31.7% of the root canals to be negative.

This study is the first to use T-RFLP to investigate the composition of the root canal microflora. T-RFLP is most commonly used for systematic community analysis of environmental samples (Marsh 1999, Kitts 2001). The advantage of T-RFLP is a higher throughput and reproducibility in monitoring bacterial communities (Osborn *et al.* 2000, Blackwood *et al.* 2003). In addition, T-RFLP was found to be very reproducible. A drawback of this technique however, is the sometimes limited discriminatory power, as different species can present with the same TRF. Nevertheless, this approach allowed for the detection of bacterial species never previously found in endodontic infections by culture and was in agreement with the findings of other reports using molecular techniques (Conrads *et al.* 1997, Siqueira *et al.* 2000, Jung *et al.* 2001, Rolph *et al.* 2001).

Conclusion

T-RFLP, a DNA-based nonculture-dependent approach made possible the rapid assessment of the bacterial biodiversity of necrotic pulps and filled root canals with apical periodontitis and revealed the presence of bacteria that have rarely been described in connection with this pathosis. Compared with other, culture-based studies, which usually indicate monoinfection of root filled canals associated with post-treatment disease, a higher diversity in these samples was found. No statistical differences with regard to biodiversity could be assessed between root canals with necrotic pulps and filled root canals. In root filled teeth, a significantly higher mean number of TRFs per sample was detected in samples with defective coronal restorations.

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References

Baele M, Baele P, Vaneechoutte M et al. (2000) Application of tDNA-PCR for the identification of enterococci. *Journal of Clinical Microbiology* **38**, 4201–7.

Bhaskar SN (1966) Periapical lesions – types, incidence and clinical features. *Oral Surgery Oral Medicine Oral Pathology* **21**, 657–71.

Blackwood CB, Marsh T, Kim S-H, Paul EA (2003) Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. *Applied and Environmental Microbiology* **69**, 926–32.

Byström A, Claesson R, Sundqvist G (1985) The antibacterial effect of camphorated paramonochlorphenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals. *Endodontics and Dental Traumatology* **1**, 170–5.

Cheung GSP, Ho MWM (2001) Microbial flora of root canal-treated teeth associated with asymptomatic periapical radiolucent lesions. *Oral Microbiology and Immunology* **16**, 332–7.

Conrads G, Gharbia SE, Gulabivala K, Lampert F, Shah HN (1997) The use of a 16S rDNA directed PCR for the detection of endodontopathogenic bacteria. *Journal of Endodontics* **23**, 433–8.

Dahlén G, Haapasalo M (1998) Microbiology of apical periodontitis. In: Ørstavik D, Pitt Ford TR, eds. *Essential Endodontology. Prevention and Treatment of Apical Periodontitis*, 1st edn. Oxford, UK: Blackwell Science Publications, pp. 106–30.

Deveaux E, Hildelbert P, Neut C, Romond C (1999) Bacterial leakage of Cavit, IRM, TERM, and Fermit: a 21-day *in vitro* study. *Journal of Endodontics* **25**, 653–9.

Engebretson JJ, Moyer CL (2003) Fidelity of restriction endonucleases in determining microbial diversity by terminal-restriction fragment length polymorphism. *Applied Environmental Microbiology* **69**, 4823–9.

Fabricius L, Dáhlen G, Öhman AE, Möller AJR (1982) Predominant indigenous oral bacteria isolated from infected root canals after varied times of closure. *Scandinavian Journal of Dental Research* **90**, 134–44.

Fraser JG (1974) Chelating agents: their softening effect on root canal dentin. *Oral Surgery Oral Medicine Oral Pathology* **37**, 803–11.

Hancock HH, Sigurdsson A, Trope M, Moiseiwitsch J (2001) Bacteria isolated after unsuccessful endodontic treatment in a North American population. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* **91**, 579–86.

Heuer H, Smalla K (1997) Application of denaturating gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) for studying soil microbial communities. In: Van Elsas JD, Wellington EMH, Trevors JT, eds. *Modern Soil Microbiology. New York: Marcel Dekker, pp. 353–73.*

Hommez GMG, Coppens CRM, De Moor RJG (2002) Periapical health related to the quality of coronal restorations and root fillings. *International Endodontic Journal* **35**, 680–9.

Jung I-Y, Choi B-K, Kum K-Y et al. (2001) Identification of oral spirochetes at the species level and their association with other bacteria in endodontic infections. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* **92**, 329–34.

Khayat A, Lee S-J, Torabinejad M (1993) Human saliva penetration of coronally unsealed obturated root canals. *Journal of Endodontics* **19**, 458–61.

Kitts CL (2001) Terminal restriction fragment patterns, a tool for comparing microbial communities and assessing community dynamics. *Current Issues in Intestinal Microbiology* **2**, 17–25.

Langendijk PS, Schut F, Jansen GJ et al. (1995) Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in faecal samples. *Applied and Environmental Microbiology* **61**, 3069–75.

Liu W, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphism of genes encoding 16S rRNA. *Applied and Environmental Microbiology* **63**, 4516–22.

Marsh TL (1999) Terminal restriction fragment (T-RFLP), an emerging method for characterizing diversity among homologous populations of amplification products. *Current Opinion in Microbiology* **2**, 323–7.

Molander A, Reit C, Dahlén G, Kvist T (1998) Microbiological status of root-filled teeth with apical periodontitis. *International Endodontic Journal* **31**, 1–7.

Möller AJR (1966) Microbiological examination of root canals and periapical tissues of human teeth. Methodological studies. *Odontologisk Tidskrift* **74**, 1–380.

Mombelli A, Minder CE, Gusberti FA, Lang NP (1989) Reproducibility of microscopic and cultural data in repeated subgingival plaque samples. *Journal of Clinical Periodontology* **16**, 434–42.

Munson MA, Pitt-Ford T, Chong B, Weightman A, Wade WG (2002) Molecular and cultural analysis of the microflora associated with endodontic infections. *Journal of Dental Research* **81**, 761–6.

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**, 695–700.

Nair PNR, Sjögren U, Krey G, Kahnberg K-E, Sundqvist G (1990) Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy-resistant periapical lesions: a long-term light and electron microscopic follow-up study. *Journal of Endodontics* **16**, 580–8.

Nair PNR, Pajarola G, Schroeder HE (1996) Types and incidence of human periapical lesions obtained with extracted teeth. *Oral Surgery Oral Medicine Oral Pathology* **81**, 93–102.

Osborn AM, Moore ER, Timmis KN (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community

structure and dynamics. *Environmental Microbiology* **2**, 39–50.

Ray HA, Trope M (1995) Periapical status of endodontically treated teeth in relation to the technical quality of the root filling and the coronal restoration. *International Endodontic Journal* **28**, 12–8.

Rolph HJ, Lennon A, Riggio MP et al. (2001) Molecular identification of microorganisms from endodontic infections. *Journal of Clinical Microbiology* **39**, 3282–9.

Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y (2003) Application of terminal RFLP-analysis to characterize oral bacterial flora in saliva of healthy subjects and patients with periodontitis. *Journal of Medical Microbiology* **52**, 79–89.

Saunders WP, Saunders EM (1994) Coronal leakage as a cause of failure in root-canal therapy: a review. *Endodontics and Dental Traumatology* **10**, 105–8.

Siqueira JF, Rocas IN, Oliveira JCM, Santos KRN (2000) Detection of *Treponema denticola* in endodontic infections by 16S rRNA gene directed polymerase chain reaction. *Oral Microbiology and Immunology* **15**, 335–7.

Siqueira JF, Rôças IN, Moraes SR, Santos KRN (2002) Direct amplification of rRNA gene sequences for identification of selected oral pathogens in root canal infection. *International Endodontic Journal* **35**, 345–51.

Siren EK, Haapasalo MPP, Ranta K, Salmi P, Kerosuo ENJ (1997) Microbiological findings and clinical treatment procedures in endodontic cases selected for microbiological investigation. *International Endodontic Journal* **30**, 91–5.

Sjögren U, Sundqvist G, Nair PNR (1995) Tissue reaction to gutta-percha particles of various sizes when implanted subcutaneously in guinea pigs. *European Journal of Oral Sciences* **103**, 313–21.

Socransky SS, Gibbons RJ, Dale AC, Bortnick L, Rosenthal E, MacDonald JB (1963) The microbiota of the gingival crevice in man. 1. Total microscopic and viable counts and counts of specific organisms. *Archives of Oral Biology* **8**, 275–80.

Suau A, Bonnet R, Sutren M et al. (1999) Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Applied and Environmental Microbiology* **65**, 4799–807.

Sundqvist G, Figdor D, Persson S, Sjögren U (1998) Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* **85**, 86–93.

Tronstad L, Asbjørnsen K, Døving L, Pedersen I, Eriksen HM (2000) Influence of coronal restorations on the periapical health of endodontically treated teeth. *Endodontics and Dental Traumatology* **16**, 218–21.

Vasquez AS, Ahrne B, Petterson B, Molin G (2001) Temporal temperature gradient gel electrophoresis (TTGE) as a tool for identification of *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus zea* and *Lactobacillus rhamnosu*. *Letters of Applied Microbiology* **32**, 215–9.