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Assessment of intraradicular bacterial composition by terminal restriction fragment length polymorphism analysis

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Background: The aim of the study was to assess the bacterial community structures associated with endodontic infections using terminal restriction fragment length polymorphism (T-RFLP), and to investigate the correlation of whole community profiles with the manifestation of particular clinical features.
Methods: Intraradicular samples were collected from 34 subjects and classified into three study groups based on the observed clinical symptoms: acute (n = 16), sub-acute (n = 8), and asymptomatic (n = 10). Genomic DNA was extracted from each sample, submitted to polymerase chain reaction using a fluorescently labeled 16S ribosomal DNA forward primer, and digested with two tetrameric endonucleases (*Hha*I and *Msp*I). The terminal restriction fragments (T-RFs) were subsequently discriminated in an automated DNA sequencer, and the results were filtered using a statistics-based criterion.
Results: Totals of 138 (*Hha*I) and 145 (*Msp*I) unique T-RFs were detected (means 13.1 and 11.9) and there was high inter-subject variability in the bacterial assemblages. Odds-ratio analysis unveiled the existence of higher order groups of positively associated T-RFs, restating the concept that intricate ecological relationships may take place in the root canal space. A significantly greater T-RF prevalence was detected in acute cases, suggesting a straight correlation between species richness and spontaneous pain.
Conclusion: Overall, no T-RFLP profile representing a specific bacterial consortium could be associated with the manifestation of symptoms of endodontic origin.

Key words: 16S ribosomal DNA; bacteria; community structure; endodontic infection; root canal; terminal restriction fragment length polymorphism

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Over the years, substantial efforts have been taken towards characterization of the root canal microbiota, either by cultivation or by molecular-based approaches. Even though only a selected subset of oral bacteria is capable of invading the pulp chamber and establishing endodontic infection, the infected intraradicular ecosystem still harbors sufficient microbial complexity to warrant further investigations by contemporary methodologies (36, 43, 44). It is now known that the unfolding of specific signs and symptoms of endodontic origin can be attributed to particular bacterial species in the intraradicular milieu (10, 11, 13, 17, 24). Moreover, bacterial interactions occur inside the root canal relatively frequently (41, 55) and special combinations of bacteria have been shown to trigger synergistic pathogenic effects (9, 13). In light of these findings, it becomes reasonable to assume that the root canal microbial assemblages can also encompass a relevant role in the establishment of features of clinical importance.

The application of molecular ecology tools in the study of endodontic infections can provide a more comprehensive view of root canal microbial communities, allowing for the assessment of potential relationships between community structure and particular sets of clinical parameters (46, 52). One major goal of microbial

community structure analysis is the assessment of species diversity, which is generally defined by two fundamental parameters: species richness (the number of species in the community) and species evenness (the quantity of each species). Assessment of these variables in endodontic infections might be impaired by cultivation techniques because approximately 50% of oral bacteria cannot grow under artificial conditions (1). In this scenario, community structure analysis should ideally be conducted using techniques capable of describing the very fastidious and non-cultivable fraction of the oral microbial consortium.

Terminal restriction fragment length polymorphism (T-RFLP) offers high-throughput quantitative information on community structure and dynamics in complex environments (3, 6, 39). In brief, the technique employs polymerase chain reactions (PCR) targeting the bacterial 16S ribosomal RNA gene (16S rDNA), in which one of the markers is fluorescently labeled at its 5′ end. The PCR product is digested with tetrameric endonucleases, and the terminal restriction fragments (T-RF) are measured by an automated DNA sequencer. This generates a genetic fingerprint of the bacterial composition for each polymicrobial sample (29, 32). More recently, computer-based tools for virtual amplification and digestion of DNA, profile filtering, alignment of fragments, multivariate statistical analysis, and taxonomic prediction of fragments have been developed, bringing additional analytical value to the technique (2, 48, 50).

T-RFLP has already been used for contrasting the bacterial composition of saliva from healthy and periodontal patients (47), monitoring shifts in the subgingival microbial profiles after treatment (45), investigating the effect of coronal restoration quality and of radiotherapy on endodontic bacterial composition (19, 20), and for comparing the bacterial communities of intraradicular samples and pus aspirates from symptomatic and asymptomatic teeth (46).

In this study, the T-RFLP technique was applied to assess the intraradicular bacterial composition of acute, sub-acute, and asymptomatic cases of endodontic infections, and to compare the community structures of these clinical categories.

Material and methods

Subjects and specimens

Working approval was granted by the Ethics Committee of the Piracicaba Dental School, State University of Campinas and written consent was obtained from each of the participants. Twenty-four patients (14 men and 20 women) ranging from 15 to 61 years old (mean 34.6 years, SD 13.3) were selected from those referred for endodontic treatment at the Piracicaba Dental School. A thorough medical and dental inventory was obtained from each of the participants. Teeth included in the study consisted of incisors, canines, premolars, and molars harboring pulpal necrosis and no previous history of root canal treatment (primary endodontic infections). Cases were mostly consequent to advanced *de novo* caries lesions, or to secondary caries associated with faulty restorations. Teeth were examined by visual inspection, probing, thermal stimulation, vertical and lateral percussion, palpation, and by preoperative radiographic evaluation. A negative response to thermal stimulation, absence of pulpal bleeding after coronal access, and the presence of periapical radiolucency, fistula, purulent drainage, or swelling were the foremost clinical parameters considered for the diagnosis of pulpal necrosis. Cases were divided into three study groups for statistical correlation with T-RFLP patterns. Group I (acute) consisting of symptomatic cases presenting moderate to severe spontaneous pain, associated or not with periapical radiolucency or swelling; Group II (sub-acute) consisting of cases presenting at least one of the following: mild to moderate pain on occlusion, tenderness on vertical or lateral percussion, and/or sensitivity on palpation, but no spontaneous pain; Group III (asymptomatic) consisting of asymptomatic chronic infections associated with periapical radiolucency (17).

Intraradicular sample collection

Each patient was submitted to local anesthesia and the tooth was isolated from the oral cavity with a rubber dam. The crown was cleaned to eliminate food debris and dental plaque. Antisepsis of the crown and operation field was conducted with 2.5% sodium hypochlorite for 1 min, followed by inactivation with 5% sodium thiosulfate (38). Coronal access to the cavity was gained with a high-speed bur irrigated with sterile saline solution. As the pulp chamber was reached, a sterile #15 K-file was introduced into the root canal at 3 mm from the root apex. After careful instrumentation, the handle of the K-file was cut off and its active portion was placed in a test tube containing 1 ml TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Four sterile #15 paper points were introduced consecutively into the canal for 20 s each and placed in the same test tube. Samples were kept on ice and immediately transported to the laboratory.

DNA extraction

The DNA from the clinical samples and reference bacteria were extracted as described elsewhere (44), resuspended in 30 μl TE buffer, and stored at −20°C for further analyses.

Primer set selection

The PRIMER SEQUENCE PREVALENCE ANALYSIS program (MICA3; http://mica.ibest.uidaho.edu) was used to verify the range of detection of all possible combinations of the primers 27F, 63F, 1389R, 1392R, and 1492R against the 16S rDNA Ribosomal Database Project II Release 9.37, with one mismatch allowed within 10 bases from the 5′ end of either primer. The primer set 63F (5′-CAGGC CTAACACATGCAAGTC-3′) and 1389R (5′-ACGGGCGGTGTGTACAAG-3′) (39) revealed the highest number of positive matches, and therefore was chosen for subsequent T-RFLP analyses. The 63F primer was labelled with 6′-carboxy-fluorescein at its 5′ end to allow fluorescent detection of the terminal restriction fragment.

Amplification of DNA

One hundred microliter reactions were performed with 60 ng DNA, 1 × PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs (Promega, Madison, WI, USA), 0.4 μM primers (IDT Technologies, San Jose, CA, USA), and 1.0 U *Taq* DNA polymerase (Invitrogen Co., Carlsbad, CA, USA), according to the following temperature profile: initial denaturation at 94°C for 3 min; 25 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2 min; final extension at 72°C for 10 min. Aliquots (3 μl) of each PCR product were submitted to 1.0% agarose gel electrophoresis and stained with ethidium bromide. PCR products were purified with the QIAquick PCR Purification kit (Qiagen, Valencia, CA, USA) as stated by the manufacturer, and the DNA was quantified in a spectrophotometer at 260 nm.

Endonuclease restriction of amplified DNA

A 200 ng sample of each purified product was restricted independently with *Hha*I (G'CGC) and *Msp*I (C'CGG) endonucleases (35) in 15-μl reactions, as instructed by the manufacturer (New England Biolabs Inc, Ipswich, MA, USA). Aliquots of the original PCR products were left undigested and used as negative controls for T-RFLP analysis.

Generation of T-RF profiles

Two microliters of each restricted PCR product and the respective negative control were mixed with 7 μl deionized formamide and 1 μl MapMarker 1000 ROX size standard (Bioventures Inc., Murfreesboro, TN, USA), denatured at 95°C, and immediately placed on ice. Mixtures were injected for 30 s in replicates into an ABI 3100 Sequence Analyzer (Applied Biosystems – Hitachi, Foster City, CA, USA). Raw T-RF profiles were generated by GeneScan 3.7 software (Applied Biosystems) using the Local Southern Method.

Filtering and binning of T-RF profiles

Filtering of raw T-RF profiles was accomplished using the tools from IBEST (2). In brief, the standard deviation of the T-RF profiles from each individual sample was determined, and T-RFs with peak areas lower than a 10 standard deviation threshold value were considered as analytical noise and removed from the profiles. Filtered T-RFs were binned with T-Align software (53) using a 1.0 confidence value. Consensual T-RF profiles of replicate samples were generated by computing the mean fluorescence intensities of corresponding T-RFs among replicates, and by discarding peaks that were detected in only one of the replicates. Absolute peak heights were transformed into percentage values, as a way to level the total fluorescence among samples after the removal of solitary peaks by T-Align.

Data analysis

Statistical analysis was performed considering each T-RF as an individual operational taxonomic unit (OTU). T-RF prevalence and the Chao1 non-parametric species-richness estimator were determined with EstimateS 8.0 (http://purl.oclc.org/estimates). The hypothesis that T-RF richness was not statistically different among the study groups was assessed with the Mann–Whitney *U*-test with significance level $\alpha = 0.05$. A command-line software (Fragmate) was designed at our laboratory to assess differences in T-RF prevalence among the study groups based on a two-tailed Fisher's exact test with $P \leq 0.05$, and considering T-RF presence and absence as test parameters. The software was also used to investigate positive associations among all possible pairs of T-RFs, as determined by the odds-ratio statistics (OR). An OR value for each T-RF pair was obtained by calculating the odds of finding the effector fragment simultaneously with a target fragment, divided by the odds of finding the effector without it (18, 41, 55). OR values ≥ 2.0, with a 95% confidence interval not including 1.0, were considered an indication of positive associations among the tested T-RFs. Finally, a stringent furthest neighbor clustering algorithm was applied on all T-RF pairs to investigate the existence of higher order groups of positively associated T-RFs. This algorithm ensured that all fragments within a group harbored at least an OR = 2.0 among themselves. A Bray–Curtis dissimilarity matrix from a combined *Hha*I and *Msp*I peak height data set was used for hierarchical clustering analysis of community profiles with unweighted pair group method with arithmetic mean (UPGMA) by MEGA 4 (http://www.megasoftware.net), and for principal coordinates analysis (PCoA) by PrCoord 1.0 and Canoco 4.5 (Biometris, Wageningen, the Netherlands). A quantitative display of relative peak heights from T-Align consensus profiles was obtained with Cluster 3.0 and TreeView 1.1 (http://rana.lbl.gov). Taxonomical prediction of the most prevalent T-RFs, and of positively associated fragments, was inferred by TAP-T-RFLP (33) based on a 16S rDNA sequence database of oral bacteria (TRFMA; http://myamagu.dent.kyushuu.ac.jp/bioinformatics/trfma/index.html) (37), adopting a maximum error interval of two nucleotides.

Results

All samples were positive for bacteria, as indicated by PCR with universal 16S rRNA gene primers FAM-63F and 1389R. T-RFLP analysis revealed a total of 447 *Hha*I and 405 *Msp*I T-RFs in 34 cases of endodontic infections, with 138 and 145 unique T-RFs, respectively. T-RF prevalence per subject ranged from 1 to 27 for *Hha*I, and from 1 to 24 for *Msp*I endonucleases (means 13.1 and 11.9 respectively) with an overall average of 12.5 T-RFs. Additional information on T-RF prevalence is presented in Table 1. Group I displayed significantly higher *Hha*I ($P = 0.0001$) and *Msp*I ($P = 0.0005$) T-RF prevalence, while Group II displayed a significantly lower *Msp*I T-RF prevalence ($P = 0.004$), compared with the asymptomatic group. To downweight

Table 1. Terminal restriction fragment (T-RF) distribution in 34 cases of endodontic infections (S1–S34)

	*Hha*I	*Msp*I	Average
*Group I. acute (*n = *16)*			
S1	8	7	7.5
S3	12	16	14.0
S4	20	14	17.0
S6	11	9	10.0
S7	16	10	13.0
S9	9	13	11.0
S12	19	16	17.5
S13	11	14	12.5
S16	24	20	22.0
S17	25	23	24.0
S20	16	13	14.5
S21	26	24	25.0
S23	22	16	19.0
S24	27	23	25.0
S29	3	3	3.0
S33	0	3	1.5
Total	249	224	236.5
Distinct	106	101	103.5
Singletons[1]	56	54	55.0
Twins[2]	21	19	20.0
Mean	15.6	14.0	14.8
*Group II. sub-acute (*n = *8)*			
S5	22	19	20.5
S10	16	14	15.0
S14	22	16	19.0
S26	3	1	2.0
S27	20	12	16.0
S30	3	1	2.0
S31	1	3	2.0
S34	2	1	1.5
Total	89	67	78.0
Distinct	66	53	59.5
Singletons[1]	50	42	46.0
Twins[2]	11	9	10.0
Mean	11.1	8.4	9.8
*Group III. asymptomatic (*n = *10)*			
S2	18	24	21.0
S8	8	8	8.0
S11	16	17	16.5
S15	14	17	15.5
S18	21	14	17.5
S19	14	10	12.0
S22	15	14	14.5
S25	1	2	1.5
S28	1	5	3.0
S32	1	3	2.0
Total	109	114	111.5
Distinct	67	79	73.0
Singletons[1]	44	56	50.0
Twins[2]	12	16	14.0
Mean	10.9	11.4	11.2
Overall Total	447	405	426.0
Overall distinct	138	145	141.5
Overall singletons[1]	57	60	58.5
Overall twins[2]	27	38	32.5
Overall mean	13.1	11.9	12.5

[1]T-RFs exclusively detected in one subject; [2]T-RFs exclusively detected in two subjects.

potential biases as a result of differences in study group sizes, the Chao1 non-parametric species richness estimator was computed, taking into consideration T-RF presence/absence data. According to this analysis, Group I of symptomatic infections harbored a greater number of estimated T-RFs when compared with the asymptomatic group. Differences were markedly higher for *Hha*I T-RFs, and more discrete for *Msp*I T-RFs (Table 2).

Highly frequent fragments detected in more than 25% of the subjects were taxonomically predicted by TAP-TRFLP (Fig. 1). These included *Hha*I T-RF sizes 57 (*Prevotella* sp. oral clone AU069), 59 (*Pedobacter* sp. oral clone AV100/*Flavobacterium-like* sp. oral clone AZ123), 62 (*Campylobacter sputorum*), 64 (*Tannerella forsythia* oral clone BU063), 341 (*Pseudoramibacter alactolyticus*), 509 (*Lactobacillus* sp.), 514 (*Fusobacterium nucleatum*), 550 (*Selenomonas sputigena*), and 556 (*Veillonella* sp. oral clone ASCB03); and *Msp*I T-RF sizes 52 (*Capnocytophaga sputigena*), 56 (*Tannerella forsythia*), 57 (*Prevotella* sp. oral clone AU069), 183 (*Clostridiales* bacterium oral clone P4PA_66 P1, *Actinomyces* sp. oral clone EP053), 245 (*F. nucleatum*), 266 (*Actinomyces* sp. oral clone AP064), 267 (*Veillonella* sp./*Selenomonas* sp./*Actinomyces* sp.), 450 (*Eubacterium* sp. oral clone DO008), and 464 (*Peptostreptococcus* sp. oral clone CK035) (Fig. 1). No single T-RF was statistically more prevalent in either of the study groups, as inferred by the Fisher's exact test. To assess positive associations among T-RFs, the OR statistics between all possible pairs of T-RFs were determined. This analysis revealed 17 *Hha*I T-RF pairs and eight *Msp*I T-RF pairs harboring OR ≥ 2.0 (Table 3). These T-RF pairs were clustered into higher order consortiums by FRAGMATE with a furthest neighbor algorithm (Table 4).

Hierarchical clustering analysis of combined *Hha*I and *Msp*I whole community profiles exhibited no evident tendencies for clustering according to the predefined clinical parameters (Fig. 2). Likewise, multivariate PCoA did not reveal any distinct grouping of samples as a function of the study groups (Fig. 3). The heat map of T-ALIGN consensus T-RF profiles of all study subjects is shown in Fig. 1, along with the taxonomic identification of most prevalent T-RFs.

Table 2. Chao1 richness estimator values per study group

Study group	Chao 1
*Hha*I	
Group I (acute)	180.7
Group II (sub-acute)	179.6
Group III (asymptomatic)	139.8
Overall	189.6
*Msp*I	
Group I (acute)	177.7
Group II (sub-acute)	151.0
Group III (asymptomatic)	169.6
Overall	196.8

Discussion

The T-RFLP technique allows rapid determination of community structure of complex microbial communities, constituting a reliable tool for detecting major differences in microbial composition among samples or study groups (3, 6, 16, 28). This technique has proved to be of great value in the study of various types of oral infections from contrasting clinical conditions (19, 20, 45–47).

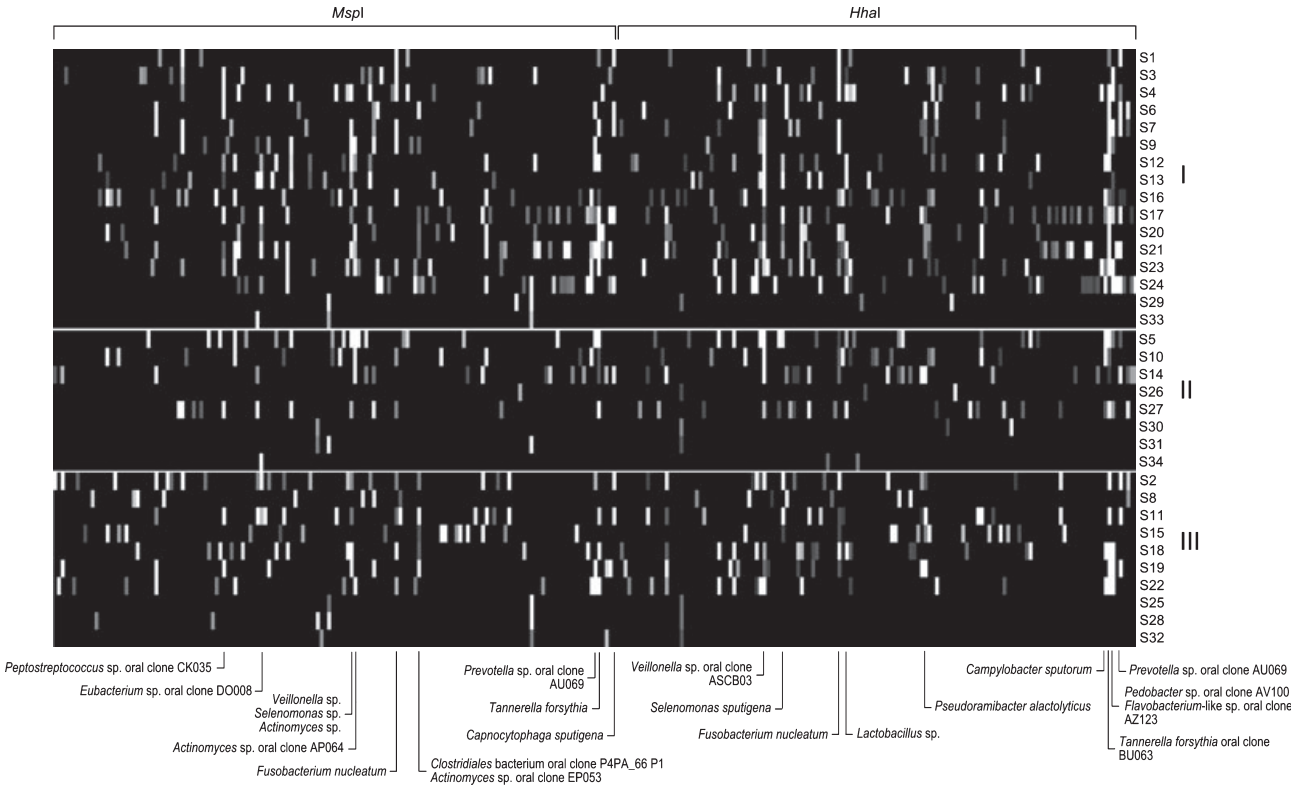

Fig. 1. Quantitative display (heat map) of relative terminal restriction fragment peak fluorescence obtained with *Hha*I and *Msp*I endonucleases from 34 cases of endodontic infections, along with taxonomical prediction of terminal restriction fragments detected in more than 25% of the subjects. Subjects are grouped according to the observed clinical parameters: I (acute), II (sub-acute), III (asymptomatic). Filled squares, low fluorescence; open squares, high fluorescence.

Table 3. Taxonomic identity of positively associated terminal restriction fragment (T-RF) pairs, as predicted by TAP-T-RFLP

T-RF	Effector OTU	T-RF	Target OTU	OR	95% CI	
*Hha*I						
55	*Capnocytophaga* sp.	57	*Capnocytophaga sputigena*	2.1	0.76	0.76
		341	*Pseudoramibacter alactolyticus*	2.1	0.76	0.76
57	*Capnocytophaga sputigena*	556	*Veillonella* sp. oral clone ASCB03	4.3	1.46	1.46
		514	*Fusobacterium nucleatum*	7.7	2.03	2.03
59	*Pedobacter* sp. oral clone AV100/	62	*Campylobacter sputorum*	4.9	1.60	1.60
	Flavobacterium-like sp. oral clone AZ123	514	*Fusobacterium nucleatum*	2.1	0.74	0.74
		556	*Veillonella* sp. oral clone ASCB03	8.7	2.17	2.17
62	*Campylobacter sputorum*	514	*Fusobacterium nucleatum*	4.6	1.52	1.52
		556	*Veillonella* sp. oral clone ASCB03	41.8	1.77	5.69
174	*Propionibacter* sp. oral clone HE018	336	*Neisseria* sp. oral clone AP132	2.1	0.76	0.76
		514	*Fusobacterium nucleatum*	2.1	0.76	0.76
340	*Serratia marcescens/Rothia* sp.	556	*Veillonella* sp. oral clone ASCB03	2.8	1.01	1.01
341	*Pseudoramibacter alactolyticus*	514	*Fusobacterium nucleatum*	5.8	1.75	1.75
		556	*Veillonella* sp. oral clone ASCB03	5.8	1.75	1.75
509	*Lactobacillus* sp.	556	*Veillonella* sp. oral clone ASCB03	4.9	1.59	1.59
514	*Fusobacterium nucleatum*	556	*Veillonella* sp. oral clone ASCB03	7.2	1.98	1.98
550	*Selenomonas sputigena*	556	*Veillonella* sp. oral clone ASCB03	4.1	1.42	1.42
*Msp*I						
57	*Prevotella* sp. oral clone AU069	183	*Clostridiales* bacterium oral clone P4PA_66 P1/*Actinomyces* sp. oral clone EP053	2.2	0.79	0.79
		267	*Veillonella* sp./*Selenomonas* sp./*Actinomyces* sp.	4.1	1.42	1.42
58	*Tannerella forsythia* oral clone BU063	266	*Actinomyces* sp. oral clone AP064	2.8	1.01	1.01
		461	*Haemophilus* sp. oral clone BJ095/*Neisseria* sp. oral clone AK105	2.8	1.01	1.01
100	*Bifidobacterium* sp.	392	*Burkholderia* sp. oral strain C37KA	2.8	1.01	1.01
259	*Selenomonas* sp. oral clone JI021	450	*Eubacterium* sp. oral clone DO008	2.1	0.76	0.76
266	*Actinomyces* sp. oral clone AP064	461	*Haemophilus* sp. oral clone BJ095/*Neisseria* sp. oral clone AK105	2.7	0.99	0.99
433	*Campylobacter* sp. oral clone BB120	464	*Peptostreptococcus* sp. oral clone CK035	2.1	0.76	0.76

95% CI, 95% confidence interval (should not include 1.0); OTU, operational taxonomic unit.
Taxa corresponding to a single T-RF are separated by a slash (/). T-RFs are given in nucleotide base values.

Table 4. Higher order groups of positively associated terminal restriction fragments (T-RFs), as determined by FRAGMATE

Groups	OTUs
*Hha*I	
59, 62, 514, 556	*Pedobacter* sp. oral clone AV100/*Flavobacterium* sp. oral clone AZ123, *Campylobacter sputorum*, *Fusobacterium nucleatum*, *Veillonella* sp. oral clone ASCB03
57, 514, 556	*Capnocytophaga* sp., *Fusobacterium nucleatum*, *Veillonella* sp. oral clone ASCB03
62, 514, 556	*Campylobacter sputorum*, *Fusobacterium nucleatum*, *Veillonella* sp. oral clone ASCB03
341, 514, 556	*Pseudoramibacter alactolyticus*, *Fusobacterium nucleatum*, *Veillonella* sp. oral clone ASCB03
*Msp*I	
58, 266, 461	*Tannerella forsythia* oral clone BU063, *Actinomyces* sp. oral clone AP064, *Haemophilus* sp. oral clone BJ095/*Neisseria* sp. oral clone AK105

OTU, operational taxonomic units.
Taxa corresponding to a single T-RF are separated by a slash (/). T-RFs are given in nucleotide base values.

Previous reports have demonstrated that more than one bacterial species can be assigned to a specific T-RF with a particular restriction enzyme (4, 6, 37). Nonetheless, bacterial species that yield identical T-RFs with one endonuclease may generate unique T-RFs with another. Hence, using an additional enzyme can substantially increase analysis accuracy, providing satisfactory resolution powers for most T-RFLP assays (4, 6). A tangible number of T-RFLP studies on clinical and environmental microbiota has been performed with one or two endonucleases, with successful discrimination of natural groups of samples (8, 15, 19, 20, 45, 47). *Hha*I and *Msp*I endonucleases, in particular, hold sufficient discriminative power for taxonomical prediction of fragments (35), for assessing oral bacterial diversity (45), and for comparing the subgingival microbiota from periodontally treated and untreated subjects (47).

A recent *in silico* survey on commonly used primers for clinical studies has demonstrated that no broad-range 16S rDNA primer pair is capable of covering all bacterial phyla (23). Election of a universal primer set should therefore rely on the judicious observation of taxa predominantly found in the target microenvironment. In this study, primers 63F and 1389R were chosen because of their broader range of detection when compared with other widely used sets, as determined by a computer-assisted assay. The use of this primer pair also facilitates taxonomic prediction of T-RFs because both lie nested to the annealing positions of primers 27F and 1392R/1492R/1525R, broadly used for 16S rDNA PCR amplification and sequencing (22, 39). Nonetheless, some groups of bacteria possess a 3′ mismatch at the 63F primer annealing site that can prevent proper PCR amplification. This limitation is not observed when DNA from a pure culture is used as template (30), but may hold importance when applied to heterogeneous DNA (22). Even so, primers 63F and 1389R may provide amplification efficiencies similar to those of 27F and 1492R for members of *Bacteroidaceae*, *Clostridia* and *Proteobacteria* under PCR conditions similar to

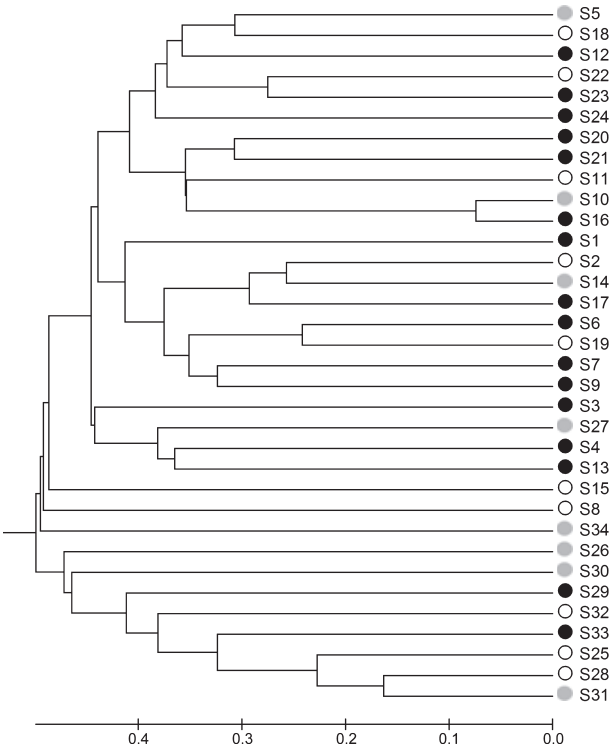

Fig. 2. Unweighted pair group method with arithmetic mean (UPGMA) dendogram of terminal restriction fragment length polymorphism profiles from 34 cases of endodontic infections. A combined *Hha*I and *Msp*I data set was used to obtain a peak height sensitive Bray–Curtis dissimilarity matrix. Filled circles, acute symptoms; gray circles, sub-acute symptoms; open circles, asymptomatic.

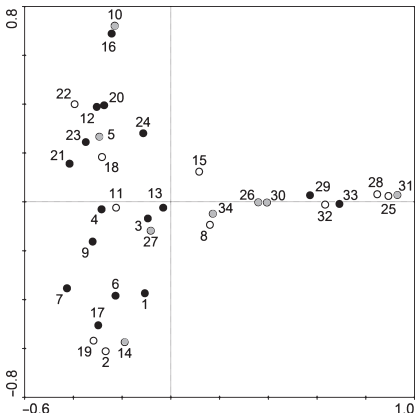

Fig. 3. Principal coordinates analysis plot (first and second axes) of terminal restriction fragment length polymorphism profiles obtained from 34 cases of endodontic infections. A combined *Hha*I and *Msp*I data set was used to obtain a peak height sensitive Bray–Curtis dissimilarity matrix. Filled circles, acute symptoms; gray circles, sub-acute symptoms; open circles, asymptomatic.

those used herein and, therefore, suited the general purposes of the study.

A statistically based filtering methodology was applied to refine the raw T-RFLP data (2). This approach sets a specific cut-off value for each sample based on the standard deviation of total fluorescence levels. According to this methodology, averages of 13.1 *Hha*I T-RFs and 11.9 *Msp*I T-RFs were detected per subject. Our results have a high degree of concordance with those of a previous investigation by Sakamoto et al. (46), who detected averages of 13.1 *Hha*I T-RFs and 14.3 *Msp*I T-RFs in 11 cases of endodontic infections using a cut-off based on peak area. Conversely, our results are substantially higher than those achieved by Hommez et al. (19, 20), who detected averages of 6.6, 5.8, and 6.2 *Bst*UI T-RFs in intraradicular samples from various clinical conditions using a peak height cut-off.

By considering each T-RF as a unique OTU, species diversity indexes can be extended to T-RFLP analysis and statistical inference on samples and study groups can be performed from an ecological standpoint (2, 6, 48). The Chao1 diversity index is a continuous-by-sample type, non-parametric, species-richness estimator (5). It provides prediction of species richness in a sample set, assuming that the number of unseen species is related to the prevalence of singleton and twin species. Higher Chao1 species-richness values were inferred for Group I (acute infections) with both endonucleases (Table 2). Moreover, a significantly higher T-RF prevalence was observed in Group I when compared with the asymptomatic group (*Hha*I: $P < 1.0 \times 10^{-4}$, *Msp*I: $P = 5.0 \times 10^{-4}$ by the Mann–Whitney test). Viewed coordinately, the results suggest a straight correlation among the number of bacterial species residing in the intraradicular milieu and the manifestation of endodontic symptoms. Similar results were obtained in a T-RFLP study by Sakamoto et al. (46).

No single T-RF could be detected in all subjects, and a high variability in the bacterial profiles was observed, irrespective of study group. This was primarily conceived by the observation of a substantial number of singletons (T-RFs present in only a single subject) and twins (T-RFs present in only two subjects): *Hha*I digestion disclosed a total of 84 singletons plus twins, while *Msp*I revealed 98 singletons plus twins, corresponding to 61% and 68% of the total unique T-RFs, respectively (Table 1). These results indicate that a subset of the endodontic microbiota may correspond to infrequent taxa, corroborating the results of previous cultivation and molecular-based investigations in that a high variability in the microbial assemblages can be noted among individuals (13, 44, 52).

In spite of the high variability detected among T-RF profiles, nine *Hha*I and nine *Msp*I T-RFs were found in more than 25% of the study subjects, composing a hypothetical common bacterial consortium. We have used a computer-based approach for predicting these 18 highly frequent T-RFs (Fig. 2) based on a 16S rDNA sequence database of oral bacteria (37). The use of a restricted data set helped us to minimize much of the imprecision commonly associated with taxonomic prediction of T-RFs (6, 37). All the predicted OTUs represented non-cultivated clones or species previously detected in the root canal, subgingival plaque, or saliva, with the exception of *Eubacterium* sp. oral clone DO008, *Pedobacter* sp. oral clone AV100, and *Veillonella* sp. oral clone ASCB03, respectively reported in samples from tongue dorsa (27), advanced noma lesions (40), and aphthous ulcers (31).

Nutritional interdependences and bacterial coaggregation are the two foremost ecological interactions among oral bacteria (12). In the root canal, a chain model was proposed, according to which essential nutrients required by certain bacterial populations are provided as metabolic

products by others in the community (56). OR has been the most widely employed statistical system for assessment of interactions among pairs of oral bacteria, with an OR ≥ 2.0 considered as indication of positive associations among taxa (13, 18, 26, 41, 54, 55). In the present study, positive associations between several pairs of T-RFs were assessed by OR analysis with very narrow confidence intervals (ranges approaching zero), indicating a high precision in values (Table 3). Such intricate interactions seemed to be preponderantly orchestrated by sets of two to three taxonomic units, some of which composed core groups capable of participating in more than one higher-order consortium (Table 4).

Of the 23 positively associated T-RFs identified, special attention was drawn to *Veillonella* sp. oral clone ASCB03 and to *F. nucleatum*, both displaying markedly higher frequencies in association events. However, because *Veillonella* sp. oral clone ASCB03 was not detected as an effector phylotype, the observed associations could partially be a reflection of its high prevalence in the sample set (20/34). Still, a strikingly high OR (41.8) between this phylotype and *C. sputorum* was detected. Complementary studies should ideally be performed to validate the true potential of *Veillonella* sp. oral clone ASCB03 for cellular interactions *in vitro*; nonetheless, the nutritional demands of this particular phylotype are as-yet undetermined (31). *F. nucleatum* is a notorious root canal inhabitant, whose prevalence may reach up to 48% by cultivation (13, 41, 55). In this study, *F. nucleatum* was highly frequent (17/34) and displayed elevated associative potentials both as target and as effector OTU. Positive associations among *F. nucleatum* and other endodontic species have been previously reported in a cultivation study (55). It has also been shown that this species is capable of coaggregating with other oral bacteria *in vitro* (49). The synergistic activities of *F. nucleatum* might be partially the result of its highly versatile metabolism, and of the excretion of formate, which may serve as a nutritional factor for other gram-negative anaerobes (12, 25, 42, 56).

Hierarchical clustering analysis with UPGMA of whole community T-RF profiles displayed no evident clades with a predominance of representatives from a single study group, and no clearly distinct clusters of samples as a function of the study groups could be discriminated by multivariate analysis with PCoA. Within the methodological boundaries of the study, the results indicate that no specific bacterial consortium is directly correlated with the presence of symptoms. The results also suggest that the bacterial factors potentially involved in pain production may not be intrinsic to a particular consortium of species. In reality, common bacterial components such as lipopolysaccharides, lipoteichoic acid, and peptidoglycans are widespread among many bacterial groups, and are well known for their proinflammatory and algogenic properties (14). Some of these factors have been shown to play crucial roles in periapical inflammation (7) and bone resorption (21). As a result of the polymicrobial nature of endodontic infections, a diversity of virulence factors may be encountered in the root canal, which may activate different inflammatory pathways simultaneously, introducing additional complexity to the study of odontogenic symptoms. Moreover, the immune response elicited in the periapical tissues is a very intricate biological process (34, 51), and many of the specific interactions among microbial factors and the patient's endogenous components still require further clarification. In all, the results highlight the importance of considering host factors as additional analysis parameters in epidemiological studies on endodontic infections.

The intraradicular bacterial assemblages associated with acute, sub-acute, and asymptomatic endodontic infections was assessed using T-RFLP. Analysis of T-RF profiles revealed a high variability in the intraradicular bacterial composition among subjects, irrespective of clinical parameters. Positive associations were found among selected groups of T-RFs, restating the concept that intricate ecological interactions can arise in the intraradicular environment. In addition, a significantly higher number of T-RFs was observed in acute cases, suggesting a straight correlation between species richness and spontaneous pain. Nonetheless, no specific T-RFLP profile reflecting a particular bacterial consortium could be directly correlated with the manifestation of symptoms of endodontic origin.

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