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Bacterial community profiles of endodontic abscesses from Brazilian and USA subjects as compared by denaturing gradient gel electrophoresis analysis

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This study compared the bacterial community profiles of the microbiota associated with acute apical abscesses from Brazilian and USA patients using denaturing gradient gel electrophoresis (DGGE). DNA was extracted from purulent exudate aspirates and part of the 16S rRNA gene was amplified by polymerase chain reaction and separated by DGGE. The resulting banding patterns, which were representative of the bacterial community structures in samples from the two locations, were then compared. Distinct DGGE banding patterns were observed from different samples. Ninety-nine bands with distinct positions in the gels were detected, of which 27 were found only in the USA samples and 13 were exclusive to Brazilian samples. Four of the 59 shared bands showed very discrepant findings with regard to prevalence in the two locations. Cluster analysis of DGGE banding profiles showed a great variability in the bacterial populations associated with teeth with abscesses regardless of the geographical location. Two big clusters, one for each location, were observed. Other clusters contained a mixture of samples from the two locations. The results of the present study demonstrated a great variability in the bacterial community profiles among samples. This indicates that the bacterial communities of abscesses are unique for each individual in terms of diversity. The composition of the microbiota in some samples showed a geography-related pattern. Furthermore, several bands were exclusive for each location and others were shared by the two locations and showed great differences in prevalence.

Key words: acute apical abscess; endodontic microbiology; molecular biology; 16S rRNA gene

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In a broad sense, apical periodontitis can be regarded as a group of inflammatory diseases with a multitude of clinical features caused by microorganisms infecting the root canal system and/or the periradicular tissues (2). Acute apical abscess is a variation of the disease and figures as a challenge for emergency treatments, particularly when the host defenses are unable to confine the infection to the proximities of the apical foramen and the infection spreads through the bone. These situations may require systemic antibiotic therapy and the choice of the most eligible antimicrobial drug should rely on susceptibility testing of the microorganisms isolated. However, it is more commonly based on the most probable microorganisms and previous susceptibility testing (1). Standard protocols have been established on the basis of epidemi-

ological data and are proposed to treat these infections worldwide, but recent findings showing differences in the prevalence of several species between distant geographic locations (3, 29) may put any kind of standardization into question.

Data from epidemiological microbiology studies carried out in some geographic regions have been intuitively considered applicable to other distinct locations, with the clear idea that 'everything is everywhere and playing the same role'. Nevertheless, studies have recently fostered the idea that oral microbial communities can differ according to their geographic location (10–12, 38). Differences in microbial communities are mainly the result of variation in the relative abundances of taxa, including the presence of a particular taxon in one assemblage and its absence from another (17). This is arguably a result of differences in host-related and/or environmental selective pressures that operate in the same type of habitat but in distant locations. It has been shown that the prevalence of some species in endodontic samples can significantly vary between patients from different locations (3, 29). Nevertheless, it still remains to be determined whether these variations are restricted to certain species or involve the whole profile of the bacterial communities.

Recent culture-independent studies investigating the breadth of bacterial diversity in the oral cavity have revealed that more than 50–60% of the bacterial taxa found in different oral sites are as yet uncultivated phylotypes (14–16). When the diversity of the endodontic microbiota was assessed, it was found that 40–55% of the taxa represent as yet uncultivated bacteria (18, 27). Therefore, a comprehensive study of the bacterial populations in endodontic infections requires the application of culture-independent methods that overcome most of the limitations of the culturing methods. The profile of bacterial communities in a given environment can be assessed using methods such as culturing approaches, 16S rRNA gene clone library analysis, and genetic fingerprinting techniques. Although the clone library analysis has been widely used to decipher microbial diversity in different human sites (5, 6, 15, 23), the approach is usually time-consuming, labor intensive and expensive, being virtually impractical for multiple sample analysis (30). Genetic fingerprinting techniques can be used as alternatives to the cloning approach because they provide a profile representing the genetic diversity of a microbial community and allow the simultaneous analysis of multiple samples, making it possible to compare the diversity of different communities. Denaturing gradient gel electrophoresis (DGGE) has been one of the most commonly used techniques when fingerprinting microbial communities (20, 21). In DGGE, polymerase chain reaction (PCR) -generated DNA fragments of the same length but with different nucleotide sequences can be separated. The DGGE approach takes into account both the cultivable and the hitherto uncultivated portions of the microbial community under analysis. This method has been recently used to evaluate bacterial community profiles in different types of endodontic infections and/or for detection of novel uncultivated phylotypes in these infections (24, 32, 35).

As part of our ongoing efforts to analyse the geographic influence on the composition of the endodontic microbiota, this study was undertaken to compare the bacterial community profiles of the microbiota associated with acute apical abscesses from patients in Brazil and the USA.

Material and methods

Subjects and sample collection

This study was carried out in accordance with guidelines of the Ethics Committee at the Estácio de Sá University and the Institutional Review Board at the Oregon Health & Science University. The material examined was taken from adult patients who were seeking emergency treatment at the Department of Endodontics, Estácio de Sá University, at three hospitals in Rio de Janeiro, Brazil, or at the School of Dentistry, Oregon Health & Science University, Portland, OR. The teeth involved showed carious lesions, necrotic pulps and radiographic evidence of periradicular bone loss. Diagnosis of acute apical abscess was based on the presence of spontaneous pain, exacerbated by mastication, and localized or diffuse swelling, along with fever, lymphadenopathy, or malaise. No apparent communication from the abscess to the oral cavity or the skin surface was observed. No teeth showed significant gingival recession and there was an absence of periodontal pockets deeper than 4 mm.

Samples were taken by aspiration of the purulent exudate from the swollen mucosa over each abscess as previously described (3, 31). Eighteen samples were from Brazilian patients and 17 other samples were from USA patients.

DNA extraction

One hundred microliters of each clinical sample was used for DNA extraction. Samples were thawed to 37°C for 10 min and spun for 30 s. Afterwards, the microbial suspension was pelleted by centrifugation for 10 min at 5000 g. The pellet was then resuspended in 180 µl buffer ATL supplied by QIAamp DNA Mini Kit (Qiagen, Valencia, CA) and 20 µl proteinase K (20 mg/ml) was added. Samples were incubated for 3 h at 56°C. Next, 200 µl of ethanol was added and DNA was isolated by adding the lysate to the Qiagen columns as described by the manufacturer. Finally, the total bacterial DNA was eluted with 200 µl AE buffer (Qiagen). DNA extracts were stored at −20°C.

PCR amplification

A 16S rRNA gene fragment corresponding to nucleotide positions from 968 to 1401 (*Escherichia coli* numbering) was amplified using the following universal bacterial primers: 968f (5′-AAC GCG AAG AAC CTT AC-3′) containing a 40-base pair (bp) GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) added to its 5′-end, which makes it suitable for DGGE, and 1401r (5′-CGG TGT GTA CAA GAC CC-3′). Primers were as described by Nübel et al. (22).

The PCR mixture comprised 5 µl of the supernatant from clinical samples, 25 pmol universal primers, 5 µl of 10X PCR buffer (Biotools, Madrid, Spain), 3.8 mM $MgCl_2$, 2.5 U of *Tth* DNA polymerase (Biotools), 0.2 mM concentration of each deoxynucleoside triphosphate (Biotools) and sterile ultrapure water to a final volume of 50 µl. Negative controls consisting of sterile ultrapure water instead of sample were included with each batch of samples analysed.

PCR amplification was performed in a DNA thermocycler (Mastercycler Personal, Eppendorff, Hamburg, Germany). The temperature profile included an initial denaturation step at 94°C for 2 min, followed by 35 cycles of a denaturation step at 94°C for 1 min, a primer annealing step at 55°C for 1 min, an extension step at 72°C for 2 min and a final step of 72°C for 10 min. Before the DGGE analysis, the presence of PCR products was confirmed by electrophoresis in a 1.5% agarose gel conducted at 4 V/cm in Tris–borate–ethylenediaminetetraacetic acid (EDTA) buffer. The gel was stained for 15 min with 0.5 µg/ml ethidium bromide and viewed

under 300-nm wavelength ultraviolet light. A 100-bp DNA ladder digest (Biotools) served as the molecular size standard.

DGGE assay

DGGE of PCR products generated with the 968f-GC/1401r primer set was performed using the Dcode Universal Mutation Detection System (Bio-Rad Dcode, Richmond, VA) at 75 V and 60°C for 16 h in 0.5X TAE buffer [20 mM Tris–acetate (pH 7.4), 10 mM sodium acetate, 0.5 mM disodium EDTA]. The PCR products (30 µl) were loaded on 6% (w/v) polyacrylamide gels containing a linear gradient of the denaturants urea and formamide, increasing from 40% at the top of the gel to 70% at the bottom [100% denaturant corresponded to 7 M urea and 40% (v/v) formamide]. Three gels were run to accommodate all the samples. In each gel, samples were loaded in alternate slots according to the geographical location (Fig. 1). A 10-ml stacking gel without denaturant was added on top. A sample showing multiple bands in earlier experiments was loaded in the first and last slots of each gel to facilitate alignment and comparison between gels. After electrophoresis, gels were stained with SYBR green I nucleic acid gel stain (Molecular Probes, Leiden, the Netherlands) for 40 min and then scanned using a Storm PhosphorImager (Amersham Biosciences, Uppsala, Sweden).

DGGE analysis

Individual lanes of the DGGE gel images were straightened and aligned using ADOBE PHOTOSHOP (Adobe Systems, Inc. Mountain View, CA). The DGGE banding patterns were converted to a binary matrix using presence–absence data. Cluster analysis by Ward's method or by an unweighted pair group method using arithmetic averages (UPGMA) following calculation of Euclidean distances or Pearson coefficient, respectively, was conducted to determine whether the samples revealed a non-random pattern and to compare DGGE banding patterns.

Results

Genomic DNA extracted from abscess samples was amplified by universal bacterial primers directed towards the V6-to-V8 regions of the 16S rRNA gene. PCR amplicons were detected in all samples, which indicated that bacteria were always present and that the PCR were conducted without significant amounts of inhibitors in clinical samples. Negative controls yielded no bands, which demonstrated that contaminants were not present. In addition, the fact that no band occurred in all samples examined confirmed that PCR reagents were free of contaminants.

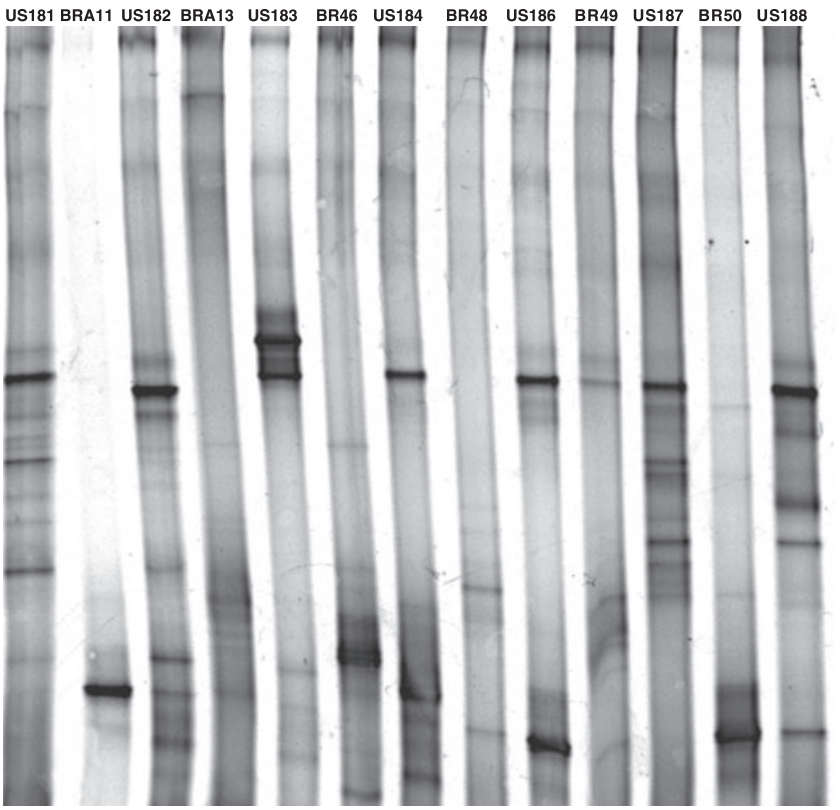

Fig. 1. Representative gel showing DGGE profiles of amplified 16S rRNA gene from acute apical abscess samples of patients from two geographical locations.

A representative DGGE gel is displayed in Fig. 1. Most DGGE profiles contained intense DNA bands, as well as many faint DNA bands. A few profiles consisted almost exclusively of faint bands. No single band occurred in all profiles, but the most prevalent band was found in 25 of the 35 samples examined. This band was very frequent regardless of the sample origin, being present in 12/17 (71%) of the USA samples and in 13/18 (72%) of the Brazilian samples.

Distinct banding patterns were observed from different samples. Comparative analysis of the two data sets revealed bands that were common for samples taken from the two locations, but about 40% of the DGGE bands appeared to be unique for each sample origin. Ninety-nine bands with distinct positions in the gels were detected, of which 27 were found only in the US samples and 13 were exclusive to Brazilian samples. Of the 59 bands shared by samples from the two locations, four bands showed very discrepant findings with regard to prevalence. Two of them were much more frequent in US samples (29% vs. 6% and 35% vs. 6%) while the other two were more common in Brazilian samples (33% vs. 12% and 17% vs. 6%). The mean diversity of bands detected in the 16S rRNA gene community profiles were 7.2 ± 4 (range 1–20) for Brazilian samples and 10.4 ± 3.4 (range 5–19) for USA samples.

Cluster analysis of DGGE band polymorphism showed a great variability in the bacterial populations associated with tooth abscesses regardless of their geographical location. Two big clusters of interest were evident: one composed of 10/18 Brazilian samples (with one USA 'crasher' sample) and the other composed of 7/17 USA samples (with one Brazilian 'crasher' sample) (Fig. 2). The other clusters observed comprised mixtures of samples from the two locations, with no clear dominance of either.

Discussion

Molecular methods have innumerable advantages over culture procedures, including higher sensitivity, higher specificity and the ability to detect hitherto uncultivated bacteria (30). One additional

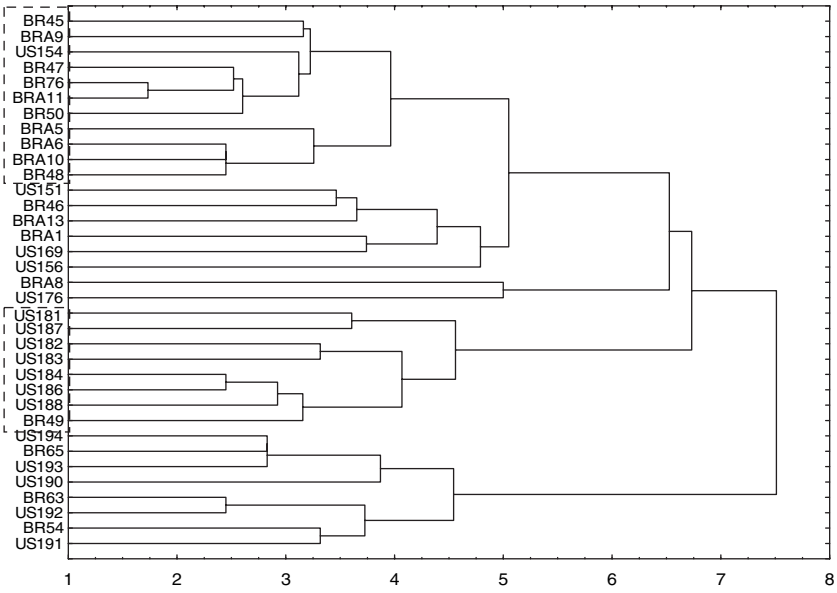

Fig. 2. Dendrogram obtained by Ward's method for clustering of DGGE patterns of samples taken from acute apical abscesses of Brazilian (BR prefix) or US (USA prefix) patients. Two clusters were almost exclusively composed of samples from the same location.

advantage, which was exploited in this study, concerns the fact that DNA can be extracted from samples and stored frozen before being submitted for analysis as one batch in distant laboratory facilities; this also enables samples to resist long-distance transportation (30). Based on this, all PCR-DGGE procedures were performed in one laboratory to avoid the biases that are inevitably introduced to an experiment when samples are analysed in different laboratories by different personnel.

Theoretically, each band in the gel would represent a single taxon, although several factors can influence this interpretation (36). While heteroduplex formation and occurrence of multiple copies of the 16S rRNA gene with sequence microheterogeneity might increase the number of DGGE bands, many other factors should reduce it, including differential spatial distribution of bacteria as a result of homogenization procedures, differential DNA extraction, PCR biases, and co-migration of bands on DGGE gels (36). Therefore, it is difficult to ascertain the precise number of species in a sample by the DGGE protocol used here. However, the bacterial community patterns are not expected to be significantly affected by these factors and DGGE has been one of the most used molecular fingerprinting methods in analysis of bacterial community structures in environmental and human samples (19, 41).

Geographical differences in the prevalence of putative endodontic pathogens had already been suspected when comparing results from individual studies performed in different locations. For instance, discrepant findings have been reported from isolate studies for the prevalence of black-pigmented anaerobic species (4, 34, 39), *Fusobacterium nucleatum* (8, 25, 33, 39) and *Treponema denticola* (7, 13, 26, 37) in samples from different countries. The reason for those differences may have been the different microbial identification methods used, but recent studies assessing samples from different locations using the same method in a single laboratory have raised the possibility that the differences are more likely to be related to the study populations (3, 29). Although the main focus of the present investigation was on the overall picture of bacterial communities without identifying bacteria, the profiles of whole communities showed that differences in the prevalence of species are rather clear. About 40% of the DGGE bands were not shared by the two groups of samples analysed, being exclusive to their respective locations. This strongly suggests that each region may show significant differences in bacterial diversity. Furthermore, as far as shared bands are concerned, markedly divergent prevalence values were observed for some of them. This confirms the discrepant findings for some species that were observed in previous studies of endodontic infections (3, 29).

The differences in occurrence and prevalence of certain species in endodontic infections are expected to influence the whole composition of the microbiota. Analysis of the similarities among the whole bacterial communities that were present in the examined samples was performed by clustering approaches. Cluster analysis involves the search for a natural grouping and can be very useful to identify samples that generate similar DGGE patterns (9). The Ward's or UPGMA dendrogram demonstrated a great interindividual variability in the DGGE band profiles representing bacterial communities, regardless of their geographic location. No profile was completely similar between samples, indicating that each individual harbors a unique microbiota. Even so, similarities in the band profiles generated two large clusters, each being almost exclusively composed of samples from a given location. One clade clustered similar band profiles from seven USA samples, while the other had similar band profiles from 10 Brazilian samples. This implies that specific samples, whether derived from USA patients or Brazilian patients, are related to each other based on the DGGE banding pattern. It also suggests that more than one profile is associated with this clinical condition and that perhaps there are a number of specific microbiotas associated with different geographic locations.

The composition of the human oral microbiota of a specific region may be influenced by a myriad of demographic and socioeconomic factors, race and immunological features, quality of community water supplies, feeding habits, climate conditions and aspects related to the use of antimicrobial agents for therapeutic purposes and/or in animal husbandry (2, 3, 28, 38, 40). Because endodontic infections are endogenous infections caused by oral microorganisms, geography-related variations observed in the oral microbiota are expected to be reflected in the bacterial communities participating in endodontic infections.

In conclusion, the results of the present study demonstrated a subject-specific variation in the bacterial community profiles, regardless of the geographic origin. Moreover, the composition of the microbiota in some samples showed a geography-related pattern. Several bands were exclusively detected in samples from either location and others were shared by the two locations showing great differences in prevalence values. Further studies should focus on the causes of geographic differences in the composition of the endodontic microbiota in different clinical conditions. Also, it

remains to be elucidated whether the observed differences in bacterial community profiles influence the outcome of standard local and systemic therapeutic procedures.

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