Objective. The purpose of this investigation was to examine the microbiota of infected root canals by using a molecular genetic method.

Study design. The presence and levels of 42 bacterial species were determined in 28 root canal samples by using whole genomic DNA probes and checkerboard DNA-DNA hybridization. To confirm the presence of bacterial DNA in clinical samples, a polymerase chain reaction with an ubiquitous bacterial primer was undertaken.

Results. The results of the checkerboard DNA-DNA hybridization analysis showed that 22 of the 42 DNA probes tested were reactive with 1 or more samples. The number of bacterial species in the root canal samples ranged from 1 to 17 (mean, 4.7). Seventeen of the 28 root canal samples were positive for at least 1 DNA probe. The most prevalent species found were as follows: Bacteroides forsythus (39.3% of the cases); Haemophilus aphrophilus (25%); Corynebacterium matruchotii (21.4%); Porphyromonas gingivalis (17.9%); and Treponema denticola (17.9%).

Conclusions. The microbiologic data of the present investigation indicated that molecular genetic methods can provide significant additional knowledge regarding the endodontic microbiota by detecting bacterial species that are difficult or impossible to culture. In addition, our findings support the current concept that endodontic infections are mixed infections of polymicrobial etiology.

Abundant scientific evidence supporting the microbial etiology of periradicular diseases has been accumulated.1,2 Earlier studies of the endodontic microbiota indicated that aerobic and facultative bacteria are dominant isolates.3,4 However, the improvements in anaerobic culturing techniques during the last 20 years have established that obligately anaerobic bacteria predominate in infected root canals.2-7

From the hundreds of microbial species that colonize the oral cavity, some potentially pathogenic bacteria have been implicated in the pathogenesis of periradicular lesions. Cultural studies have shown that species of the genera Fusobacterium, Prevotella, Porphyromonas, Eubacterium, Peptostreptococcus, Actinomyces, and Propionibacterium are more prevalent in primary root canal infections.2,5,6 Nonetheless, the prevalence of some recognized oral pathogens might have been underestimated because culture may often fail to grow fastidious anaerobic microorganisms.

During the last decade, molecular genetic methods have been used to detect microorganisms that are impossible or difficult to culture.8,9 These methods have been used to identify bacteria in clinical samples without the need for culturing and isolating them, or without biochemical tests for identification. In addition, they are more rapid than cultural methods. Thus, it is entirely possible that such molecular analytical methods can provide significant additional knowledge about the microbiota of infected root canals.

Recently, a method was introduced for hybridizing large numbers of DNA samples against large numbers of DNA probes on a single support membrane—checkerboard DNA-DNA hybridization.10 It permits the simultaneous determination of the presence of multiple bacterial species in single or multiple clinical samples. Several studies that use this molecular method have considerably enhanced the understanding of the microbiology of periodontal diseases.11-13

Based on these premises, the purpose of this study was to assess the prevalence and levels of 42 bacterial species in primary root canal infections by using checkerboard DNA-DNA hybridization.

MATERIALS AND METHODS
Specimen sampling
The examined material was selected from patients that had been referred for root canal treatment to the department of Endodontics, Estácio de Sá University,
Rio de Janeiro, Brazil. Included in this study were 28 single-rooted teeth from adult patients, all with carious lesions, necrotic pulps, and radiographic evidence of periradicular bone loss. Nine teeth were tender to percussion, and 2 other cases were diagnosed as acute periradicular abscesses. The other cases were asymptomatic. Selected teeth showed an absence of periodontal pockets greater than 4 mm. The patients' ages ranged from 18 to 60.

Samples were collected with strict asepsis. The tooth was cleaned with pumice and was isolated with a rubber dam. The tooth and the surrounding field were cleaned with 3% hydrogen peroxide and then decontaminated with a 2.5% sodium hypochlorite solution. Complete access preparations were made with sterile burs without water spray. The operative field, including the pulp chamber, was then swabbed with 2.5% sodium hypochlorite. This solution was inactivated with sterile 5% sodium thiosulfate. If the root canal was dry, a small amount of sterile saline solution was introduced into the canal. Samples were initially collected by means of a No. 15 K-type file (Maillefer, Ballaigues, Switzerland) and were used to soak up the fluid in the canal. Each paper point was retained in position for 1 minute. The file was introduced to a level approximately 1 mm short of the tooth apex, and a discrete filing motion was applied. Afterwards, 2 sequential paper points were placed to the same level and were used to soak up the fluid in the canal. Each paper point was retained in position for 1 minute. The cut file and the 2 paper points were then transferred to cryotubes containing 1 mL of 5% dimethyl sulfoxide in trypticase-soy broth (Difco, Detroit, Mich) (TSB-DMSO). Samples were immediately frozen at −20°C.

After disinfection of the oral mucosa with 2% chlorhexidine, pus from the abscessed teeth was collected by aspiration with a sterile syringe, was transferred to TSB-DMSO, and was frozen.

**DNA extraction**

The samples in TSB-DMSO were thawed at 37°C for 10 minutes and vortexed for 30 seconds. Microbial suspension was washed 3 times with 100 μL of bidistilled water by centrifugation for 2 minutes at 2500 xg. Pellets were then resuspended in 100 μL of bidistilled water, boiled for 10 minutes, and chilled on ice. After centrifugation, the supernatant was collected for testing.

**Microbiologic assessment**

*Enumeration of bacterial species in root canal samples with checkerboard DNA-DNA hybridization.*

<table>
<thead>
<tr>
<th>Acinetobacter baumannii</th>
<th>Lepotrichia buccalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomyces naeslundii</td>
<td>Neisseria mucosa</td>
</tr>
<tr>
<td>Actinomyces viscosus</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>Actinomyces odontolyticus</td>
<td>Peptostreptococcus anaerobius</td>
</tr>
<tr>
<td>Bifidobacterium dentium</td>
<td>Peptostreptococcus micros</td>
</tr>
<tr>
<td>Bartonella sp.</td>
<td>Porphyromonas gingivalis</td>
</tr>
<tr>
<td>Bacteroides forsythus</td>
<td>Prevotella intermedia</td>
</tr>
<tr>
<td>Campylobacter rectus</td>
<td>Prevotella nigrescens</td>
</tr>
<tr>
<td>Capnocytophaga gingivalis</td>
<td>Propionibacterium acnes</td>
</tr>
<tr>
<td>Corynebacterium matrachotii</td>
<td>Rothia dentocariosa</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>Ralstonia sp.</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Streptococcus oralis</td>
</tr>
<tr>
<td>Fusobacterium nucleatum vincentii</td>
<td>Streptococcus constellatus</td>
</tr>
<tr>
<td>Gemella haemolysans</td>
<td>Streptococcus gordonii</td>
</tr>
<tr>
<td>Gemella morbillorum</td>
<td>Streptococcus intermedius</td>
</tr>
<tr>
<td>Haemophilus aphrophilus</td>
<td>Streptococcus anginosus</td>
</tr>
<tr>
<td>Lactobacillus oris</td>
<td>Treponema dentico</td>
</tr>
</tbody>
</table>
from eukaryotic cells were used as a positive control for the PCR. It served to indicate the presence of bacteria in the clinical samples. Ubiquitous primer sequences were as described by Ashimoto et al.15

Aliquots of 5 μL of the supernatant from clinical samples were amplified. PCR was performed in a 50 μL of reaction mixture containing 1 μL of each primer (40 pmol), 5 μL of 10× PCR buffer, 1.25 unit Taq DNA polymerase (Gibco BRL, Gaithersburg, Md) and 0.2 mmol/L of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP) (Gibco BRL). Earlier experiments found optimal magnesium chloride concentration in the mixture to be 2.0 mmol/L.

Preparations were overlaid with 2 droplets of mineral oil and were amplified in a DNA thermocycler (PTC-100, MJ Research, Inc, Watertown, Mass). The PCR temperature profile included an initial denaturation step at 95°C for 2 minutes, followed by 36 cycles of a denaturation step at 95°C for 30 seconds, a primer annealing step at 60°C for 1 minute, an extension step at 72°C for 1 minute, and a final step of 72°C for 2 minutes. PCR products were analyzed by 1.5% agarose gel electrophoresis performed at 4 volts per centimeter in trisborate EDTA buffer. The gel was stained with 0.5 μg/mL ethidium bromide and was photographed under ultraviolet light. A 100 base pair (bp) DNA ladder digest (Gibco BRL) served as the molecular weight marker.

Data analysis

Root canal samples were individually analyzed for their content of 42 taxa by using the checkerboard DNA-DNA hybridization technique. Mean levels of each species were computed for each case. In addition, the percentage of samples containing each species was computed.

RESULTS

All 28 root canal samples contained bacteria as demonstrated by PCR with the ubiquitous bacterial primer pair. Only 1 band of the predicted size (602 bp) was present for each root canal sample (data not shown). Such results indicated that bacteria were present in all cases sampled.

The results of the checkerboard DNA-DNA hybridization analysis showed that 22 of the 42 DNA probes tested were reactive with 1 or more samples. Seventeen of the 28 samples (60.7%) were positive for at least 1 DNA probe. The most prevalent species found were as follows: Bacteroides forsythus (39.3% of the cases); Haemophilus aphrophilus (25%); Corynebacterium matruchotti.
(21.4%), Porphyromonas gingivalis (17.9%); and Treponema denticola (17.9%). Enterococcus faecalis, Capnocytophaga gingivalis, and Streptococcus intermedius were detected in 14.3% of the infected teeth sampled. Unusual oral species, such as Ralstonia sp and Pseudomonas aeruginosa were found in some cases, usually in low numbers. Escherichia coli, another unusual oral bacteria, was the predominant species in 1 case of acute abscess. Fig 1 illustrates the prevalence and levels of the species detected.

B forsythus was found in 4 teeth that showed tenderness to percussion. In 3 cases, it was the only bacterial species detected. In the other case, B forsythus was associated with P gingivalis and T denticola. Six bacterial species were detected in another tooth tender to percussion, of which P gingivalis and Peptostreptococcus micros were the predominant species. Four of the 9 teeth that showed tenderness to percussion were negative for the genomic DNA probes tested.

Mixed bacterial populations were observed in the 2 cases of acute periradicular abscesses. In 1 case, 6 bacterial species were detected in the pus sample. Eikenella corrodens was the predominant species. Seventeen bacterial species were found in the other abscessed teeth. In this case, E coli was present at high levels.

The number of bacterial species in the root canal samples ranged from 1 to 17 (mean, 4.7). Although 1 case of acute periradicular abscess contained the highest number of species detected herein, no apparent correlation was found between the number of bacterial species and signs and symptoms.

DISCUSSION

According to the current knowledge, periradicular lesions are inflammatory diseases of microbial etiology. Several different bacterial species have been associated with endodontic infections based on culture, which is considered the “gold standard” for identification of bacteria in clinical samples. In cultural studies, bacterial viability is a prerequisite, and sample handling before processing is crucial. It has recently been realized that culture underestimates many of the fastidious oral microorganisms, particularly after prolonged transportation of samples. In addition, culture fails to detect uncultivable species, which may be involved in the pathogenesis of the disease evaluated. Because DNA probes can identify bacterial species or strains difficult or impossible to culture, results obtained by DNA probes may differ from the findings obtained by cultural techniques.

Even if it is not likely that advances in molecular methods lead to a dramatic change of the picture of the endodontic microbiota, they can identify potential pathogens in periapical disease hitherto not detected by cultural methods. Applying these methods may also add new information about prevalence of some microorganisms in root canal infections. The results of the present investigation with the checkerboard DNA-DNA hybridization method demonstrated that 22 of the 42 DNA probes tested were reactive with 1 or more samples. Of particular importance was the high prevalence of some bacterial species difficult to grow, such as B forsythus and T denticola. The fastidious growth of these bacteria has hindered their study in endodontic infections. By using a 16S rDNA-directed PCR, Siqueira et al16 detected T denticola in several cases of infected root canals. B forsythus has been found in root canal infections in 2 studies.17,18 Of the 42 bacterial taxa investigated in this study, B forsythus was the most prevalent species, detected in 11 of 28 teeth (39.3% of the cases). Seven teeth were asymptomatic, and 4 showed tenderness to percussion. Because B forsythus is a recognized periodontal pathogen, its relatively high prevalence in infected root canals suggests that it can also be considered a potential pathogen in periradicular disease.

The prevalence of identified species was different compared with other studies. Of interest was the higher frequency of some bacterial species, such as B forsythus, H aphrophilus, C matruchotii, and T denticola, which have never been reported to be predominant or even be present by cultural studies. On the other hand, some bacterial species commonly found in endodontic infections by culture, such as Actinomyces species, P anaerobius, P nigrescens, P acnes, and some usual oral Streptococcus species, were not detected in this investigation. One of the probable reasons for such different findings is the different methods used. DNA probes permit the detection of difficulty-to-grow or of uncultivable species. This may have been the major explanation for the relatively high prevalence of some bacterial species. Nevertheless, DNA probes have some limitations; they may cross-hybridize with closely related microbial species, giving false results. In addition, a certain number of microorganisms must be present to allow their identification with DNA probes. The checkerboard DNA-DNA hybridization method has been reported to have a detection limit of $10^3$ to $10^4$ cells, depending on the species in question.12 It is possible that some microorganisms were present at levels <$10^4$, which was below the detection level of the DNA probes used in this study. In addition to the different methods, one should bear in mind that populations examined in different geographic locations may have been an important reason for the different bacterial prevalence observed between studies.

The checkerboard DNA-DNA hybridization method failed to detect bacteria in 11 cases. However, amplifi-
cation by PCR with an ubiquitous bacterial primer pair showed that bacteria were present in all cases examined. It is highly possible that the 42 taxa investigated were not present in these cases, though the hypothesis that they were present at levels below the detection ability of the DNA probes should not be discarded.

By using the same method used herein, Socransky et al. identified a consortium of the bacterial species P. gingivalis, B. forsythus, and T. denticola as having the highest association with periodontal disease severity, as measured by pocket depth and bleeding on probing. This microbial consortium was named red complex by those authors. In this study, this microbial complex was found in 2 cases of endodontic infections. One tooth was asymptomatic, and the other showed tenderness to percussion. In 2 other asymptomatic cases, B. forsythus and P. gingivalis were detected in association. In a previous study, we identified T. denticola in 1 of these 2 teeth by means of PCR. The failure in detecting T. denticola in this same case by the DNA-DNA hybridization method can be explained by its lower sensitivity when compared with PCR. To our knowledge, this is the first study to report the occurrence of this microbial complex in infected root canals. Because the teeth examined in this study showed absence of periodontal disease, our findings suggest that the red complex may also be involved in the pathogenesis of periradicular lesions. The actual role of this microbial complex in endodontic infections needs further investigations.

Some unusual oral species were also found, usually in low numbers and in a small number of samples. Such microorganisms included P. aeruginosa and Ralstonia sp. E coli was the predominant bacterial species in 1 case of acute periradicular abscess. Many of these microorganisms have been reported to be important infectious agents in diseases in other parts of the body, such as meningitis (P. aeruginosa and E. coli), nosocomial infections (Ralstonia sp, P. aeruginosa), as well as urinary and intestinal infections (E. coli). Many of these species have been reported to be resistant to several antimicrobial agents. Some investigators also found unusual oral species in some cases of persistent or secondary endodontic infections and in refractory periodontitis. Although particular groups of bacterial species have been associated with some forms of periradicular pathosis, no absolute association between an individual bacterial species and the signs and symptoms of an infected root canal has yet been made. The microbiologic data of this study indicated that there is a great heterogeneity among the root canal microbiota. Therefore, our findings support the current concept that endodontic infections are mixed infections of polymicrobial etiology.

REFERENCES


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