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ORIGINAL ARTICLE

On the use of denaturing gradient gel electrophoresis approach for bacterial identification in endodontic infections

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Abstract Bacteria in infected root canals of teeth evincing chronic apical periodontitis lesions were identified by a polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) approach. DNA was extracted from root canal samples, and part of the 16*S* rRNA gene of all bacteria was amplified by PCR and separated by DGGE, generating banding patterns representative of the community structure. Twenty visible bands were cut out of the gel, re-amplified, and sequenced to provide identification. Sequencing analysis revealed the presence of both cultivable and as-yet-uncultivated species in the samples analyzed, including representatives of the genera *Fusobacterium*, *Bacteroides*, *Dialister*, *Synergistes*, *Prevotella*, *Eubacterium* and *Peptostreptococcus*. Unambiguous identification was not always possible and the method's limitations are discussed. In general, the findings showed that PCR-DGGE can be useful for the identification of both cultivable and as-yet-uncultivated bacteria in endodontic infections.

Keywords Endodontic microbiology · Apical periodontitis · Uncultivated bacteria · 16*S* rRNA gene · Molecular biology

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Introduction

Apical periodontitis is an inflammatory disease of polymicrobial etiology caused by microorganisms (mainly bacteria) infecting the root canal system. Traditionally, involved microorganisms have been identified by culture techniques, but molecular biology technology has recently been applied to the investigation of the endodontic microbiota associated with different forms of apical periodontitis [3, 32]. Molecular methods can overcome several limitations of culturing approaches including the detection of as-yet-uncultivated species and more reliable identification of cultivable species [30]. As a consequence, a broader spectrum of bacterial species has been revealed in association with apical periodontitis [32].

Among molecular biology methods, polymerase chain reaction (PCR) amplification of conserved regions of the 16*S* rRNA gene, followed by cloning and sequencing of PCR products, has been widely used for deciphering the bacterial diversity in association with human healthy and diseased sites [1, 8, 12, 13, 20, 39]. Nonetheless, the cloning approach is usually time-consuming, labor intensive, and expensive, being virtually impractical for multiple sample analysis. Genetic fingerprinting techniques can be used as alternative to the cloning approach because they provide a profile representing the genetic diversity of a bacterial community from a given environment. Moreover, identification of the dominant members of the bacterial community can also be done. The denaturing gradient gel electrophoresis (DGGE) has been one of the most commonly used techniques to fingerprint bacterial communities from diverse ecosystems [18].

DGGE is an electrophoretic method that can detect differences between DNA fragments of the same size but

with different sequences. The procedure is based on the electrophoresis of PCR-amplified gene fragments in polyacrylamide gels containing a linearly increasing gradient of DNA denaturants. In addition to being applied to profile the structure of the microbiota and to determine shifts in the bacterial communities over time or after treatment, PCR-DGGE can also be used for bacterial identification purposes [6, 16, 34]. For instance, a molecular ladder can be constructed using 16*S* rRNA gene amplicons of known bacterial species and can be used in an attempt to identify target species in clinical samples by comparing band positions in the DGGE gel [9, 22]. Nonetheless, the best and more reliable way to identify bands is through the excision of the bands from the gel and further sequencing [6, 16, 38]. This approach has been used in studies of the endodontic microbiota to identify representative dominant members of some communities [22, 29] or to dissect the species diversity within some bacterial groups [33]. The purpose of this study was to apply a PCR-DGGE approach to the identification of most to all members of the endodontic bacterial communities in the root canals of teeth evincing chronic apical periodontitis lesions.

Materials and methods

Subjects and sampling procedures

Samples were taken from the root canals of eight single-rooted teeth from healthy adult patients who had been referred for root canal treatment to the Department of Endodontics, Estácio de Sá University. All teeth showed necrotic pulps, radiographic, and clinical evidence of chronic apical periodontitis and no periodontal pockets greater than 4 mm deep. Patients involved in this project have not received antibiotic therapy within the previous 2 months. The study protocol was performed in accordance with the guidelines of and after approval by the Ethical Committee at Estácio de Sá University. Sampling procedures were carried out under strict asepsis as previously described [23, 24]. Total bacterial genomic DNA was isolated according to the protocol of the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA).

PCR amplification

A 16*S* rRNA gene fragment corresponding to nucleotide positions 968–1401 (*Escherichia coli* numbering) was amplified using the following universal bacterial primers 968f (5′-AAC GCG AAG AAC CTT AC-3′) containing a 40-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. [19].

The PCR mixture comprised 5 μl of DNA extracted from clinical samples, 25 pmol of universal primers, 5 μl of 10×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, filtered milliQ water to a final volume of 50 μl. Negative controls consisted of sterile milliQ water instead of sample.

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 DGGE analysis, the presence of PCR products was confirmed by electrophoresis in a 1.5% agarose gel conducted at 4 V/cm in a Tris-borate-EDTA buffer. The gel was stained for 15 min with 0.5 μg/ml ethidium bromide and viewed under short-wavelength ultraviolet light. A 100-bp DNA ladder digest (Biotools) served as the molecular size standard.

DGGE assay

DGGE of PCR products generated with 968f-GC/1401r primer set was performed using the Dcode universal mutation detection system (Bio-Rad Dcode, Richmond, VA, USA) at 75 V and 60°C for 16 h in 0.5×TAE buffer. PCR products (30 μl) were loaded on 6% (w/v) polyacrylamide gels containing a linear gradient ranging from 30 to 70% denaturant [100% denaturant corresponded to 7 M urea and 40% (v/v) formamide] and increasing in the direction of electrophoresis. A 10-ml stacking gel without denaturant was added on top. 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).

Sequence analysis of products

Each visible band was punched out from the DGGE gel using a sterile micropipette tip, placed in 50 μl of milliQ water and left at 4°C for 24 h. A total of 5 μl of the resulting solution was added to a PCR mixture under the same PCR conditions and with the primers mentioned above. Products were then checked on an agarose gel and purified using a PCR purification system (Wizard PCR Preps, Promega, Madison, WI, USA). PCR products were sequenced directly with the 1401r primer on the ABI 377 automated DNA sequencer using dye terminator chemistry

(Amersham Biosciences). Nucleotide sequence data and electropherograms were inspected and edited using BioEdit software [10]. Sequences were corrected when obvious sequencing software errors were observed, such as when ambiguities in the sequence could be resolved according to the electropherogram. Nucleotide sequences were then compared with those available in GenBank to identify the closest relatives using the BLAST algorithm [2].

Results

DNA extracted from clinical samples was amplified using primers directed towards the V6 to V8 regions of 16*S* rRNA gene, and amplicons of the expected size were present in all samples as visualized in agarose gels. The DGGE profiles of the amplified 16*S* rRNA gene of root canal samples are shown in Fig. 1. Profiles contained both intense and faint bands ranging from one to five, and efforts were done to excise and sequence all of them. Twenty nucleotide sequences were compared and analyzed for similarities to sequences deposited in GenBank. Six bands were closest to *Fusobacterium* species/phylotypes (homologies ranging from 94 to 99%). Two bands from different cases had match values of 97 and 99% to *Bacteroides*-like sp. oral clone X083/ *Bacteroidales* oral clone MCE7_20 E1. Two bands in distinct positions in the same sample had the highest similarities to *Prevotella* oral clone F045. In another clinical sample, three distinct bands were identified as *Escherichia coli* (99% similarity). Bands 2, 8 and 19 showed the highest similarities to a *Dialister* taxon (*Dialister invisus*/ *Dialister* oral clone FY011/*Dialister* oral clone BS095), a *Peptostreptococcus* taxon (*Peptostreptococcus micros*/ *Peptostreptococcus* oral clone FG014/ *Peptostreptococcus* oral clone P4P_31_P3), and *Synergistes* oral clone BA121, respectively. One band showed low-scoring homology (96%) to the sequence of *Eubacterium* oral clone FX028. Three sequences were of poor quality because of the presence of too many ambiguous characters (Ns), and a reliable identification was not possible. Data are summarized in Table 1.

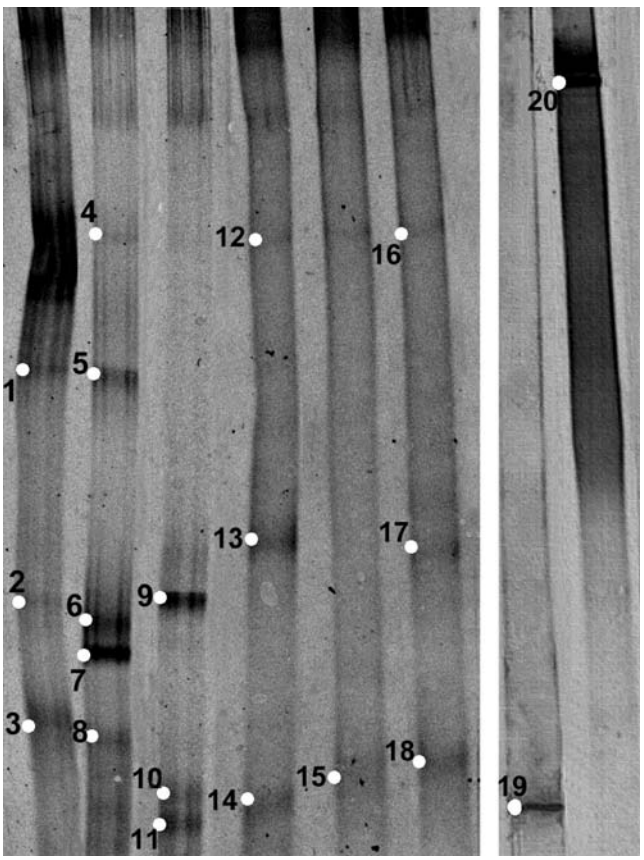

Fig. 1 DGGE profiles of amplified 16*S* rRNA gene from samples taken from infected root canals associated with apical periodontitis. Numbered bands were excised from the gels and identified by 16*S* rRNA gene sequencing analysis

Discussion

In spite of the fact that PCR-DGGE has been applied to analyze microbial communities from diverse environments, this technique has been only recently introduced in endodontic microbiology to fingerprint bacterial communities associated with different types of infection and to identify some dominant members of the communities [22, 29, 33, 34]. The rationale behind the fact that bacterial species can be distinguished by PCR-DGGE and further identified is that different bacterial species have differences in nucleotide composition within the variable regions of the 16*S* rRNA gene. Sequences present in the variable regions of the 16*S* rRNA gene are responsible for the different migration behavior of PCR products in the DGGE gel and contain the information about the species which each band belongs to.

DGGE technology is primarily used to fingerprint microbial communities in diverse environments, but it can also be used for identification. In this sense, DGGE can be of great value for bacterial identification in mixed infections, as it can detect both cultivable and as-yet-uncultivated bacteria, and in many cases, it can be a good alternative to more expensive, laborious, and time-consuming cloning procedures. In the present study, endodontic bacteria were identified after excision of the bands from the gel, re-amplification by PCR, and further sequencing. Comparison of 17 obtained sequences with sequences listed in the GenBank database revealed similarities of 94 to 99%. Several sequences showed the highest match values to sequences of members of the *Fusobacterium* genus, which have been commonly isolated from or detected in both

Table 1 Bacterial species/phylotypes detected in infected root canals by 16*S* rRNA gene-based PCR-DGGE approach

Band	Bacterial species	Similarity with nearest matches (%)	GenBank accession number
1	*Fusobacterium nucleatum*	94	AF543300/DQ440565
2	*Dialister invisus*/ *Dialister* oral clone FY011/ *Dialister* oral clone BS095	98	AY162469/AY134907/ AF287787
3	Unidentified	–	–
4	*Fusobacterium* oral clone EU021/ *Fusobacterium* oral clone AJ050/ *Fusobacterium nucleatum*	98	AF385575/ AF287805/ AE009951
5	*Fusobacterium nucleatum*	99	AF543300/DQ440565
6	*Prevotella* oral clone F045	98	AY005056
7	*Prevotella* oral clone F045	98	AY005056
8	*Peptostreptococcus micros*/ *Peptostreptococcus* oral clone FG014/ *Peptostreptococcus* oral clone P4P_31_P3	98	L60326/AF385543/ AY207059
9	*Escherichia coli*	99	AY804014
10	*E. coli*	99	AY804014
11	*E. coli*	99	AY804014
12	*Fusobacterium* oral clone EU021/ *Fusobacterium* oral clone AJ050/ *Fusobacterium nucleatum*	98	AF385575/ AF287805/ AE009951
13	*Bacteroides*-like sp. oral clone X083/ *Bacteroidales* oral clone MCE7_20 E1	99	AY005066/AF481203
14	*Eubacterium* oral clone FX028	96	AY134903
15	Unidentified	–	–
16	*Fusobacterium* oral clone EU021/ *Fusobacterium* oral clone AJ050/ *Fusobacterium nucleatum*	98	AF385575/ AF287805/ AE009951
17	*Bacteroides*-like sp. oral clone X083/ *Bacteroidales* oral clone MCE7_20 E1	97	AY005066/AF481203
18	Unidentified	–	–
19	*Synergistes* oral clone BA121	99	AY005444
20	*Fusobacterium nucleatum*	99	AF543300

asymptomatic and symptomatic endodontic infections [4, 5, 7, 11, 14, 26, 28, 35]. Some difficult-to-grow or even as-yet-uncultivated species were identified, all of which have been only recently disclosed in infected root canals by molecular approaches [15, 25, 26, 31]. Three sequences showed a low similarity (<97%) to sequences deposited in the GenBank database and could not be reliably identified. This was because of the presence of several undetermined characters in these sequences.

PCR-DGGE is a well-established molecular method that has been shown to be reliable, reproducible, rapid, and inexpensive [18]. However, as with any other method, PCR-DGGE has its own limitations. These limitations can be related either to the broad-range PCR or to the DGGE approach. The method's limitations, which can preclude the detection of some members of the community, include biases during DNA extraction [37] and differential or preferential amplification of the 16*S* rRNA gene [21, 36]. Also, only microorganisms present in relatively high concentrations are represented on the gel [17]. As a consequence, all populations present within a given habitat do not necessarily appear on DGGE banding patterns [17].

Another limitation of the 16*S* rRNA gene-based PCR-DGGE approach in analyzing the diversity of the bacterial communities is the presence of multiple copies of the 16*S* rRNA gene with sequence microheterogeneity. A single species with multiple rRNA gene copies can display a DGGE profile with multiple bands [19]. This was confirmed in the present study in which three sequenced bands from the same sample yielded sequences closely related to *E. coli*, and two bands in another sample resulted in sequences for the same species-*Prevotella* oral clone F045.

Two other reported limitations of the method were apparent in the present study and made identification difficult or even impossible for some specimens. First, because fragments to be resolved by the DGGE technique should not be longer than 500 bp, unambiguous identification is not always possible when comparing such short sequences in the databases. This limitation was apparent in this study for seven sequences obtained, for which, alternative identifications were considered. Second, comigrating bands (fragments different in sequences but with identical melting behavior) and/or bands too close one from the other to be individually retrieved from the gels can result in mixed sequences which are impossible to be identified without an additional isolation approach [6]. This second problem can be solved by the cloning of the DGGE bands after PCR re-amplification and before sequencing, and may have been responsible for the failure in identifying three of the specimens obtained in this study.

In conclusion, the broad-range nature of the PCR-DGGE method allows the detection of both cultivable and as-yet-uncultivated bacterial species [18, 27]. Furthermore, DGGE is less labor-intensive and technically difficult than anaerobic culturing procedures or cloning approaches. Multiple samples can be analyzed simultaneously on one gel, and bands of interest can be cut out and sequenced directly to identify bacterial species [17]. Reliable identification can be achieved for many bands appearing in the gel, and certain strategies can be made necessary to deal with some difficulties imposed by the technique. Thus, although the present study has not compared the PCR-DGGE approach with any "gold" standard, our findings suggest that this method can be a useful tool in the analysis of bacterial diversity in endodontic infections.

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