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ORAL MICROBIOLOGY
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Polymerase chain reaction-based denaturing gradient gel electrophoresis in the evaluation of oral microbiota

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Background/aims: Clinical evaluation of oral microbial reduction after a standard prophylactic treatment has traditionally been based on bacterial cultivation methods. However, not all microbes in saliva or dental plaque can be cultivated. Polymerase chain reaction-based denaturing gradient gel electrophoresis (PCR-DGGE) is a cultivation-independent molecular fingerprinting technique that allows the assessment of the predominant bacterial species present in the oral cavity. This study sought to evaluate the oral microbial changes that occurred after a standard prophylactic treatment with a conventional oral care product using PCR-DGGE.
Methods: Twelve healthy adults participated in the study. Pooled plaque samples were collected at baseline, 24 h after prophylaxis (T1), and 4 days after toothbrushing with fluoride toothpaste (T4). The total microbial genomic DNA of the plaque was isolated. PCR was performed with a set of universal bacterial 16S rDNA primers. The PCR-amplified 16S rDNA fragments were separated by DGGE. The effects of the treatment and of dental brushing were assessed by comparing the PCR-DGGE fingerprinting profiles.
Results: The mean numbers of detected PCR amplicons were 22.3 ± 6.1 for the baseline group, 13.0 ± 3.1 for the T1 group, and 13.5 ± 4.3 for the T4 group; the differences among the three groups were statistically significant ($P < 0.01$). The study also found a significant difference in the mean similarities of microbial profiles between the baseline and the treatment groups ($P < 0.001$).
Conclusion: PCR-based DGGE has been shown to be an excellent means of rapidly and accurately assessing oral microbial changes in this clinical study.

Key words: dental plaque; fluoride dentifrice; oral microbes; polymerase chain reaction-denaturing gradient gel electrophoresis; prophylactic treatment

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Bacteria in dental plaque have a long-standing association with oral diseases such as caries, gingivitis, and periodontitis. Bacterially induced gingival inflammation has been linked to numerous systemic sequelae (10, 11, 23, 26). Plaque control potentially can improve both oral as well as systemic health. Currently, bacterial cultivation and clinical indices are the methods commonly used in clinical studies. Bacterial cultures provide a quantitative measurement of certain pathogens present in the oral cavity, and clinical indices are used to evaluate treatment outcomes. Although quantitative assessment is essential to understand how new treatments modify bacterial colonization in the oral cavity, neither the cultivation method nor clinical examinations provide a wholesale assessment of the overall bacterial composition of dental plaque at specific or different time-points. Because of this, several new molecular culture-independent methods, such as denaturing

gradient gel electrophoresis (DGGE), have been developed. Polymerase chain reaction (PCR)-based DGGE has become one of the most frequently used techniques in studies of environmental and general microbial ecology because it can provide species profiles by differentiating PCR-amplified 16S rRNA gene segments in complex bacterial samples without cultivation (5, 6, 9, 15, 18, 19, 21, 22, 24, 33, 38).

Recently, this advanced PCR-based molecular technique has also been applied to the study of the bacterial community in the oral cavity (7, 12, 27, 28, 31, 37). PCR-DGGE provides qualitative and semi-quantitative assessment of the bacterial community in dental plaque, which may prove to be an excellent approach for gathering detailed, objective molecular data on the changes of the bacterial population in plaque during conventional clinical studies. In the present study, we report a new means of analyzing differences in bacterial profiles in dental plaque before and after a standard dental prophylactic procedure, followed by the use of fluoride toothpaste. The aim of the study was to determine the feasibility of using PCR-based DGGE approaches to monitor changes in the microbial composition of dental plaque in a clinical study.

Materials and methods

Subjects

The protocol for human subjects was approved by the safety and regulatory authorities of Colgate-Palmolive (Piscataway, NJ). Twelve healthy adults (six male and six female) with a mean age of 41.6 years voluntarily participated in this study. Participants who were antibiotic-free for the 3 months preceding the study and who had adhered to their regular oral hygiene regimen every day for at least 2 weeks before enrolment were enrolled in the study. Informed consent was obtained from each participant. Clinical examination and plaque sample collections were then performed.

Experimental design and bacterial sample collection

This single-blind study used a 5-day treatment period. All participants were asked to brush their teeth with the same toothpaste (Colgate Winter Fresh Gel, Colgate-Palmolive Company, Piscataway, NJ) for 1 week as a washout period before the trial. Three sets of pooled supragingival plaque samples were collected from each participant. On the first day of the treatment phase of the study, the first set of plaque samples was collected from the interproximal sites of all molars of each individual; this set constituted the baseline plaque sample (baseline). All participants underwent standard dental prophylaxis on the morning of evaluation and were asked to brush for 1 min with a commercial fluoride dentifrice (Colgate Cavity Protection, Colgate-Palmolive) using the same brand of toothbrush (Colgate Navigator, Colgate-Palmolive). All participants refrained from performing oral hygiene practices for 24 h and then reported back to the clinic.

On the second day of the study, a second set of pooled supragingival plaque samples was collected from the same interproximal sites of the molars of each participant; this set of samples was referred to as the 24-h plaque samples (T1) after the prophylactic treatment. All participants were asked to continue to brush their teeth with the same toothpaste for an additional 4 days. They were instructed to brush only one side of the mouth for 60 s while allowing the toothpaste foam and slurry to flow over the teeth into the unbrushed section of the mouth for an additional 30 s. On the fifth day of the study, all participants returned to the clinic for the final clinical evaluation. A third set of pooled supragingival plaque samples was collected from the interproximal sites of the unbrushed side of the mouth, and this set of samples was referred to as the 4-day postbrushing plaque samples (T4).

All plaque samples were collected with a sterile sickle scaling instrument. Each plaque sample was pooled into preweighed DNase-free and RNase-free polyethylene tubes, and the wet weight of the plaque samples was measured. All plaque samples were immediately frozen at −20°C, shipped on dry ice to the microbiology laboratory at the New York University College of Dentistry (New York, NY), and stored at −70°C for further processing.

DNA extraction

Bacterial samples were dissolved at 4°C and then washed in 1 ml of 10 mM Tris–HCl (pH 7.5) and 1 mM of EDTA buffer. The total genomic DNA of the bacterial samples was isolated with the MaterPure DNA purification kit (Epicentre, Madison, WI). An additional 10 μl proteinase K (Qiagen stock solution 10 mg/ml in TES buffer – 10 mM Tris–HCl, pH 8.0; 1 mM EDTA; 100 mM NaCl) and 2 μl mutanolysin [5000 U/ml in phosphate-buffered saline buffer (PBS)] were added to lysozyme stock solution (100 mg/ml in TES buffer) to ensure the release of DNA from all gram-positive bacteria in the plaque samples, followed by a phenol/chloroform/isoamyl alcohol extraction procedure (12, 38). DNA quality and quantity were measured with a UV-spectrophotometer at 260 nm and 280 nm (DU 640, Beckman, Hayward, CA). The initial DNA concentration was calculated by dividing the DNA in micrograms by the wet-weight plaque samples in milligrams (μg/mg). The final concentration of each DNA sample was adjusted to 10 ng/μl for all PCR applications.

PCR-DGGE assay

PCR was performed with the GeneAmp® PCR System 9700 (PE Applied Biosystems, Foster City, CA). A set of universal bacterial 16S rDNA primers, forward prbac1 (5′-CGCCCGGGGCGCGCCCCG-GGCGGGGCGGGGGCACGGGGGGA-CTACGTGCCAGCAGCC-3′) and reverse prbac2 (5′-GGACTACCAG-GGTATC-TACTAATCC-3′) (29), which targets the hypervariable V4–V5 regions of the *Escherichia coli* 16S rDNA ribosomal locus, was used with a 40-nucleotide GC-clamp (30) added to the 5′ end of prbac1. Each PCR mixture (a total volume of 50 μl) contained a standardized 100 ng the total genomic DNA, 200 μM of each dNTP, 40 pmol of each primer, 4.0 mM of $MgCl_2$, 5 μl of 10X PCR buffer II, and 2.5 U of *Taq* DNA polymerase (PE Applied Biosystems). The PCR conditions were as follows: initial denaturation at 95°C for 3 min and 35 cycles consisting of 1 min at 95°C, 1 min at 56°C, and 2 min at 72°C, plus an additional cycle of 5 min at 72°C for chain elongation. The PCR products were evaluated by electrophoresis in 1.0% agarose gels run at 60 V for 60 min, and the sizes of all amplicons (300 base pairs) were confirmed according to a molecular size standard.

A standardized 20 μl of each PCR-amplified product was loaded on the DGGE gel and separated with the Bio-Rad Dcode™ System (Bio-Rad, Hercules, CA). The same system was used for all DGGE experiments of this study. A 30–70% linear DNA denaturing gradient (100% denaturant is equivalent to 7 mol/l of urea and 40% deionized formamide) was formed in 8% (w/v) polyacrylamide gels. PCR products and species-specific DGGE standard markers (12) were directly loaded in each lane. Electrophoresis was performed at a constant 60 V at 58°C for

16 h in 1X Tris–acetate–EDTA (TAE) buffer (pH 8.5). After electrophoresis, gels were rinsed and stained for 15 min in water containing 0.5 μg/ml ethidium bromide, followed by 15 min destaining in water. DGGE images were digitally captured and recorded with the AlphaImager 3300 System (Alpha Innotech Corporation, San Leandro, CA).

Analysis of microbial profiles by DGGE

DGGE gel images were converted and transferred into a microbial profile database with Fingerprinting II Informatix™ Software (Bio-Rad). Each gel was normalized according to a DGGE standard marker, which was generated using 10 species-specific American Type Culture Collection (ATCC) type strains (12). The background was subtracted using mathematical algorithms according to the spectral analysis of overall densitometric curves. A 1.0% minimal profiling setting was used for a band search for all DGGE gels. Levels of similarity between fingerprints were calculated according to the Dice coefficient. A dendrogram was constructed from the average matrix using the unweighted pair group method by means of arithmetic averages (UPGMA). Differences in the microbial composition before and after the prophylactic treatment and application of the fluoride dentifrice were assessed by comparing the DGGE profiles of PCR-amplified 16S rDNA segments between and among the three sets of dental plaque samples. Significant differences in the number of detected PCR amplicons in the DGGE gels were determined using analysis of variance (ANOVA) and the paired-samples Student's *t*-test. Statistical analyses were performed using SPSS software (Statistical Package for the Social Sciences, version 13.0, SSPS Inc., Chicago, IL). All *P*-values <0.05 were two-tailed and considered significant.

Results

A total of 36 pooled plaque samples (in three sets) were collected from each of the 12 participants. The plaque wet-weight and the total genomic DNA per milligram of wet-weight for each clinical sample were successfully obtained and quantified (Table 1). PCR amplification was performed, and 16S rDNA fragments of the same size (300 base pairs) were obtained from all 36 samples. DGGE gels containing the three different sets of samples (baseline, T1, and T4) were obtained for each participant. A total of 12 sets (12 participants) of DGGE gel images were produced. As an illustration, eight of the 12 sets are shown in Fig. 1. The identification of PCR amplicons was performed after the normalization of the gels against the species-specific DGGE standard markers, followed by comparison of the band positions between the baseline and the T1 samples and the T4 samples by means of the fingerprinting computer program (Bio-Rad) (Fig. 2).

Based on the DGGE profile analysis, a total of 55 distinct bands were detected. The mean numbers of detected amplicons were 22.3 ± 6.1 for the baseline group, 13.0 ± 3.1 for the T1 group, and 13.5 ± 4.3 for the T4 group, respectively (Table 1). The overall differences among the three groups were statistically significant ($P = 0.008$, ANOVA) (Fig. 2). Significant differences were also found in the number of bands when the baseline group was compared with the T1 group ($P = 0.001$; paired-sample Student's *t*-test) and the T4 group ($P = 0.005$; paired-sample Student's *t*-test).

The pairwise comparisons of the DGGE fingerprint profiles were performed among the three experimental groups. An UPGMA dendrogram was constructed based on the similarity matrix (Fig. 3). The clustering analysis placed 91.6% of the baseline profiles into one dendrogram branch and all T1 and T4 profiles that were distinct from most of the baseline samples into separated clusters. The mean similarities of microbial profiles between the baseline group and the T1 and T4 groups were 0.55 ($P < 0.001$; ANOVA) and 0.53 ($P < 0.001$; ANOVA), respectively. The microbial profiles were more similar, with a mean of 0.72 between the T1 and T4 groups ($P > 0.05$; ANOVA).

Discussion

Traditionally, counting the colony-forming units on a culture plate has been used to study microbial changes before and after a clinical treatment or new oral health practice. Studies show, however, that a significant portion of the oral microflora may not grow under some experimental conditions (20, 35, 36), which could induce bias when enumerating bacterial levels in clinical studies. Recently, we reported the use of PCR-DGGE to study microbial diversity in the oral cavity (12). A significant difference in bacterial populations was observed among individuals with different caries statuses. As the DGGE technique is based on PCR amplifications of targeted fragments of the bacterial 16S rRNA gene using universal eubacterium-specific primers (19), several advantages of the DGGE technique are significant. PCR-DGGE can directly detect cultivable and noncultivable microbes in various bacterial samples and in the oral cavity (13, 17). This molecular fingerprinting tech-

Table 1. Bacterial genomic DNA extracted from clinical plaque samples and the number of fragments detected in DGGE

	Wet plaque weight (mg) ±SD			DNA concentration (μg/mg plaque) ±SD			No. of PCR amplicons detected		
Sample ID	Baseline	T1[1]	T4[1]	Baseline	T1	T4	Baseline	T1	T4
1	9.6	1.5	4.5	3167.5	1076.7	888.9	31	12	12
2	5.5	1.5	4.0	2880.7	316.7	1800.0	23	13	7
3	4.8	1.7	8.6	3947.4	3735.3	675.0	15	12	12
4	6.7	1.2	6.6	3164.2	1591.7	2310.6	25	11	14
5	10.5	0.8	5.5	3799.0	4881.3	3145.5	18	14	15
6	11.9	0.9	6.6	4684.9	2377.8	3128.8	29	17	16
7	3.3	0.6	6.1	4787.9	6716.7	1680.3	30	8	9
8	0.2	0.1	0.1	725.0	2650.0	2500.0	26	18	11
9	3.2	0.8	6.5	2062.5	2468.8	1276.9	16	10	21
10	7.5	0.8	5.2	3593.3	5525.0	1048.1	17	15	21
11	17.3	1.1	5.2	2656.1	2990.9	6134.6	14	10	13
12	4.15	0.6	3.1	6542.2	4183.3	2935.5	24	16	11
Mean ± SD	7.0 ± 4.7	1.0 ± 0.5	5.6 ± 1.5	3500.9 ± 1467.9	3209.5 ± 1878.5	2292.2 ± 1492.0	22.3 ± 6.1	13.0 ± 3.1[2]	13.5 ± 4.3[2]

[1]T1 = 24 h after treatment; T4 = 4 days after treatment.
[2]The analysis of variance for the means, $P = 0.008$ compared T1 and T4 with the baseline, respectively.

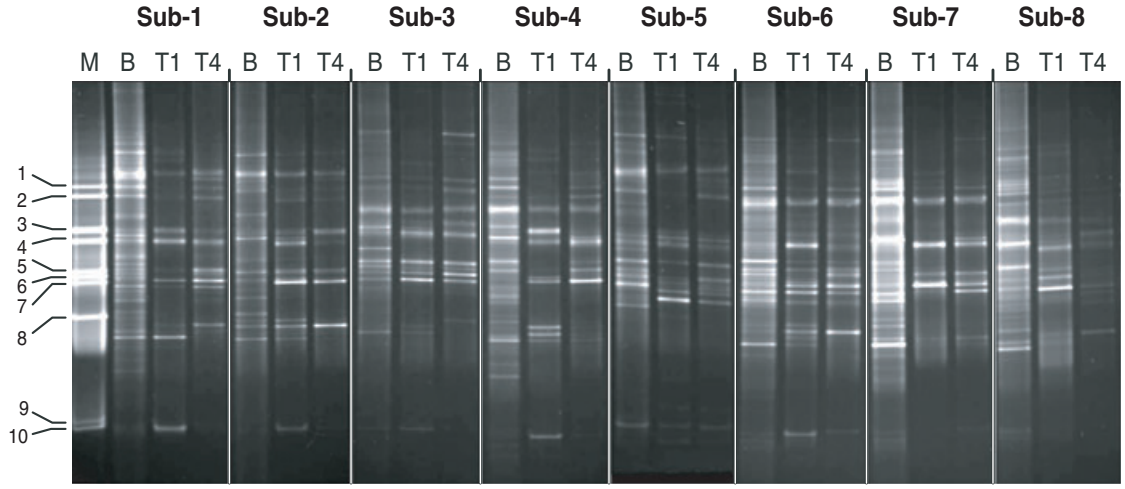

Fig. 1. Denaturing gradient gel electrophoresis (DGGE) profiles of PCR-amplified bacterial 16S rDNA segments. The gel images were from total genomic DNA of the pooled dental plaque samples of the healthy volunteers at the baseline (B), 24 h after the prophylactic treatment (T1), and 4 days after brushing (T4). A total of 12 sets (12 participants) of DGGE gel images were produced; eight of the 12 sets are included in the Figure for the purpose of illustration. The numbers of detected and dominant fragments are more pronounced among the baseline samples compared with the two post-treatment samples. DGGE reference markers are (m): 1. *Fusobacterium nucleatum* subsp. *vincenti* (ATCC49256); 2. *F. nucleatum* subsp. *nucleatum* (ATCC25586); 3. *Streptococcus sanguinis* (ATCC10556); 4. *Streptococcus oralis* (ATCC35037); 5. *Streptococcus salivarius* (ATCC7073); 6. *Streptococcus mutans* (ATCC700610); 7. *Lactobacillus paracasei* subsp. (ATCC25598); 8. *Porphyromonas gingivalis* (ATCC33277); 9. *Actinomyces odontolyticus* (ATCC17929); 10. *Actinomyces naeslundii* genospecies 1 (ATCC12104).

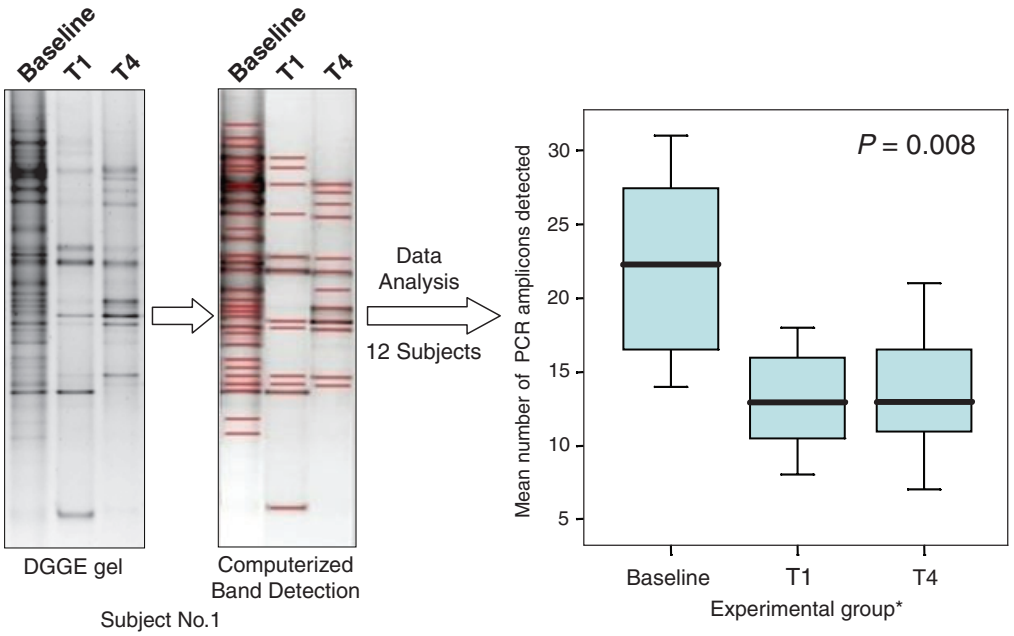

Fig. 2. DGGE data analysis. The PCR amplicons in DGGE were identified on the Fingerprinting II Informatix Software (Bio-Rad). The normalized band positions were identified for each sample, and the number of detected amplicons was summarized from all samples and analyzed using SPSS software. The data demonstrate a significant reduction in the mean number of detected amplicons after treatment, and that reduction persisted for 4 days after treatment. *Twelve subjects were included in each experimental group.

nique allows the rapid assessment of the predominant bacterial species present in complex bacterial samples such as the saliva and dental plaque (8, 12). It also allows changes in the overall microbial population over time to be monitored (14, 15, 32). Recent studies reported that more than 700 microbial species of bacteria colonize the oral cavity, of which over 50% have not been cultivated and classified at bacterial species level using conventional microbiological plating techniques (1, 16, 25). Clearly, this limits our comprehensive understanding of the microflora associated with oral diseases.

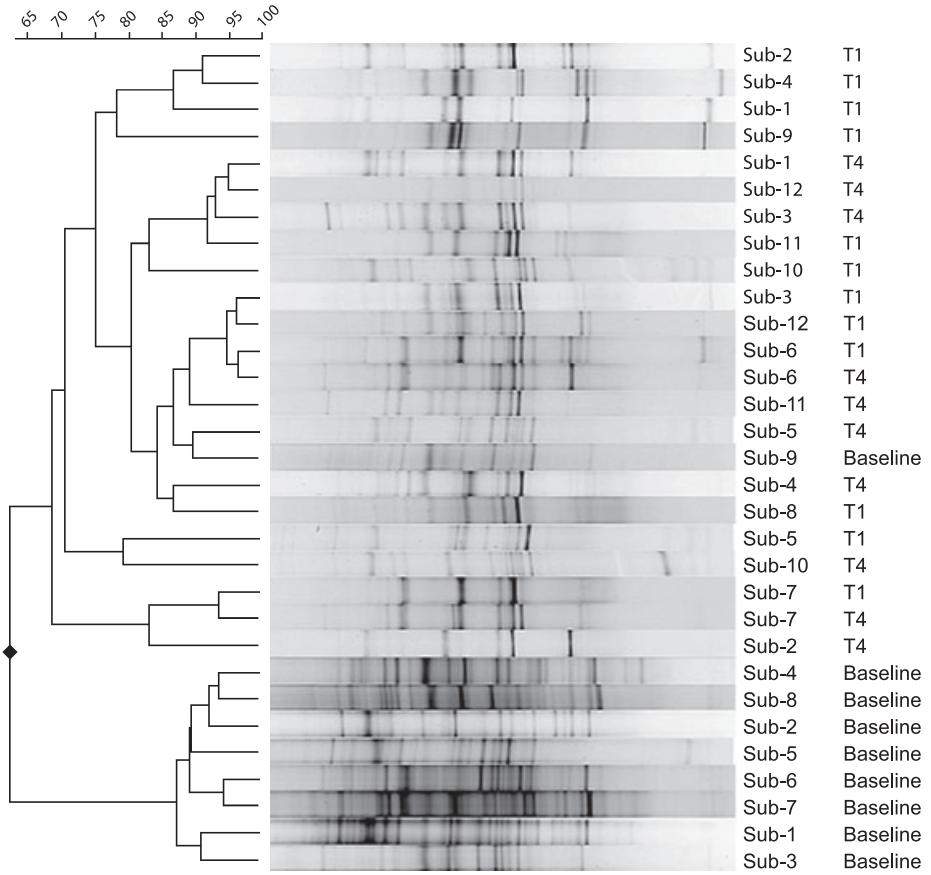

Fig. 3. The Dice coefficient for similarity among the DGGE profiles. Based on the unweighted average pair group method, 11 of the 12 baseline DGGE profiles (91.6%) were grouped into one dendrogram branch. All other T1 and T4 profiles were distinct from the baseline samples in separated clusters. Differences in the means of similarities, determined via ANOVA, were 0.55 for the baseline group vs. the T1 group ($P < 0.001$), and 0.53 for the baseline group vs. the T4 group ($P < 0.001$). The difference between the T1 and T4 groups was not statistically significant.

To determine whether the PCR-DGGE technique could be used to assess the overall microbial profile in the oral cavity and to evaluate a shift in microbial composition before and after a standard clinical intervention, the present study showed that using a set of universal primers (prbac1 and prbac2) (29), a mixture of 16S rDNA PCR products of the same size was quickly obtained from the different sets of bacterial samples; and significant changes in the total oral microbial community were observed after dental prophylactic procedures. We used prbac1 and prbac2 primers because previous research had demonstrated that a targeted V4–V5 region using this set of primers allowed PCR directly to produce a wide range of amplicons that represented great diversity of the microbial community in oral samples (29). The cluster analyses of the DGGE profiles, in particular, showed that the number of PCR amplicons present at baseline (pretreatment) was significantly reduced after the treatment. Interestingly, the reduction persisted during the study for up to 4 days, suggesting that the fluoride dentifrice might play a role in controlling bacterial growth in dental plaque. One may argue that this reduction in the numbers of detected PCR amplicons by DGGE could be a result of the mechanical removal of baseline dental plaque by toothbrushing, and that differences in band diversity detected by the fingerprinting computer program could be the result of the varying amounts of plaque samples used for DNA extraction. Notably, the plaque obtained after the 4-day brushing regimen was collected exclusively from the unbrushed side of the oral cavity, in which no mechanical plaque removal was involved. The amount of DNA used for all PCR reactions was standardized according to the wet-weight plaque samples. Thus, potential systemic bias resulting from differences in plaque sample collection was minimized.

The study also observed a higher similarity of DGGE profiles between the T1 and the T4 samples compared with the baseline profile. Our data further support previously reported findings that fluoride dentifrice may exert antimicrobial effects on both gram-positive and gram-negative anaerobes (2–4, 34). Conventional clinical indices historically have been used to document oral microbial reduction after clinical interventions. After all, the advanced PCR-DGGE technique provides molecular-based evidence of the alteration of microbial composition that can simultaneously be compared and analyzed among multiple samples of interest. The PCR-DGGE method will provide impetus to clinical investigators to further evaluate the effect of fluoride applications on overall changes in the microbial population in the oral cavity. Hence, PCR-DGGE offers a broad range of clinical applications for evaluating the effect on microbial diversity of a new treatment or a new intervention. It is particularly useful in studying diseases caused by bacterial infection, such as periodontal diseases, in

which microbial colonization and changes play a significant role in the development or reversal of the diseases.

The present study has demonstrated that PCR-DGGE is a valuable tool for clinical studies; however, we would also like to point out several limitations of this approach. First, the method is limited in its resolution and sensitivity. On average, it can detect up to 30–40 bands in a DGGE (personal communication from Dr Chris Sissons, March 2006). Second, it is based on an assumption that DNA is equally extracted from all bacterial species; any organisms representing <1% of the microbiota may not be detected by DGGE. Therefore, DGGE patterns typically represent a fingerprinting profile of PCR products from the predominant microbiota in the complex bacterial samples. Third, its reliability in determining differences in microbial community structures depends upon the quality and reproducibility of bacterial sample processing and DNA extraction. Investigators should understand these limitations when conducting clinical studies using PCR-DGGE.

In conclusion, previous evaluations of the antimicrobial effect of clinical treatments have been based mainly on the clinical measurement of plaque reduction. The discriminatory power of plaque indices varies and is subjective. The PCR-based DGGE is an advanced molecular fingerprinting technique that allows investigators to examine the colonization and distribution of predominant bacterial species present in complex samples. Results can be typically obtained within 48 h of sample collection and is less expensive compared to other molecular techniques. The findings of our study suggest that PCR-DGGE can be used as a novel analytical approach for the objective, rapid and accurate assessment of oral microbial changes in a clinical study.

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