

PCR-dipstick DNA chromatography for profiling of a subgroup of caries-associated bacterial species in plaque from healthy coronal surfaces and periodontal pockets

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ABSTRACT

The onset of plaque-mediated disease, including dental caries and periodontal diseases, is highly associated with compositional change of the resident microflora from the ecological perspective. As specific bacterial profiles have been linked to different disease stages, microbial compositional measurements might therefore have great value for clinical diagnosis. Previously we have reported a dry-reagent strip biosensor–PCR-dipstick DNA chromatography, which utilized molecular recognition of oligonucleotides and biotin-streptavidin, and the optical property of colored microspheres, for semiquantifying a five-membered subgroup of caries-associated bacterial species in supragingival plaque from healthy coronal surfaces of teeth. The present study aimed to evaluate this technique's ability to differentiate microflora by comparing the subset profiles. Sixteen subgingival plaque specimens were pooled from periodontal pockets and analyzed for the composition of *Streptococcus mutans*, *Streptococcus sobrinus*, *Scardovia wiggsiae*, *Actinomyces* sp. and *Veillonella parvula*. Detection frequencies, relative abundance of each bacterial species, and the five-membered bacterial profiles were compared between supra- and subgingival groups. The supragingival plaque harbored significantly more of the tested species and higher amount of *Actinomyces* sp. and *V. parvula*. In subgingival plaque, the predominance was obscured, since several highly overlapped profiles were found at comparable frequencies. Thus, PCR-dipstick DNA chromatography using the same plaque sample enabled simultaneous profiling of multiple species at species level and facilitated discrimination between anticipated different microflora, making this technique a promising chair-side microbiota profiling method.

The ecological plaque hypothesis (18) has revolutionized our view of the etiology of the most prevalent oral diseases, including caries and periodontal diseases. Contrary to the classical medical model,

which ascribes an infection to a single microbial pathogene, the new hypothesis suggests that a dysbiosis of the resident oral microflora, driven by an ecological perturbation, leads to plaque-mediated disease. During the transition from oral health to disease state, a dynamic interaction between the resident microflora and the habitat has been evidenced, with numerous bacterial species being implicated (2, 3, 22, 31). A substantial change of the environmental determinants could disrupt the homeostasis of the resident microflora, thereby inducing the prolifera-

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tion of putative ‘pathogenic’ bacteria (commensal bacterial species that share relevant traits that might contribute to a disease process) and resulting in an altered microbiota (30). In particular, distinct bacterial profiles resulting from this microbial community succession appear to be associated with different stages of disease (1, 22).

Concomitantly, microbiota compositional measurements for diagnosis or susceptibility assessment of polymicrobial diseases are expected to be future standard clinical practice. Modern molecular techniques, particularly those based on amplifying 16S rRNA genes, include cloning and sequencing, quantitative DNA-DNA hybridization (checkerboard), denaturing gradient gel electrophoresis (DGGE), and real-time PCR. These methods have superseded conventional culture-based methods for quantifying the microbiota of healthy and diseased oral cavity (9, 12, 13, 28). But to date, the implementation of these molecular techniques is still restricted to central laboratories because of the high equipment cost and the requirement for highly trained personnel. To realize the idea of point-of-care testing as part of routine clinical care, a test should meet the criteria of being rapid, sensitive, specific, low-cost and miniaturized (6). On the other hand, the task of defining what bacterial species are directly involved in the process of a dental disease remains tough, given that the breadth of oral bacterial diversity continues to expand. Nonetheless, accumulating research reveals that some ‘key species’ are frequently detected, with the abundance of these species exhibiting successive changes that correlate with the progression of specific dental diseases. Examples include the observation of *Streptococcus mutans*, *Actinomyces* sp., and *Lactobacillus* sp. in caries (3, 5, 21, 36), and that of *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* in periodontitis (8, 32, 34). Taken together, these data suggest an alternative way to simplify microbiota compositional measurement, namely the characterization of a ‘subset’ bacterial profiles composed of a set of discriminating bacterial species. This characterization would focus on a conserved core cluster of bacteria whose relative abundances serve to outline the whole of the oral microbiota.

In an earlier published article (35), we detailed our first attempt to apply a PCR-dipstick DNA chromatography—based on DNA biosensor technology—for rapidly measuring the abundance of five selected bacterial species in supragingival plaque samples. This dipstick strip integrated the molecular recognition of oligonucleotide strands (via biotin-streptavi-

din interactions and the optical property of blue-colored latex microspheres) with the simplicity of microfluidic assay to achieve point-of-care testing. We also proposed a semiquantitative protocol, which was based on a 10-fold dilution method and required no readout apparatus. Using this method, we succeeded in the characterization of bacterial profiles in supragingival plaque.

Bacterial composition in subgingival plaque is distinct from that in supragingival plaque (38), consistent with the suggestion that ecologically distinct conditions in the oral cavity shape the composition of microbial communities specifically adapted to different sites (22, 37). Meanwhile, previous studies demonstrated that the same bacterial species are found in both supra- and subgingival plaque, including periodontal pocket (19, 37), suggesting that microbiota in supragingival plaque influences the bacterial composition in subgingival plaque (and vice versa). Therefore, in the present study, we applied the PCR-dipstick method to subgingival plaque and compared the levels of five caries-associated bacteria with those in healthy supragingival plaque, which were reported in our previous study (35). Based on the results, we consider differences and similarities in bacterial composition between healthy supragingival plaque and periodontal pocket, as well as verifying the usefulness of this method for both supra- and subgingival plaque analyses.

MATERIALS AND METHODS

Subjects and plaque sampling. Informed consent was obtained from each subject enrolled in this study. This study was approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry, Sendai, Japan. Sixteen patients (mean age, 56.1 ± 8.4 years; range, 36–68 years) with periodontitis were randomly selected at Tohoku University Hospital. Subjects had not received periodontal treatment or antimicrobial therapy for at least 6 months prior to sampling; each was free of systemic diseases. Probing depths were measured in all teeth at six sites per tooth in each subject, and the teeth with the deepest probing depths were chosen as the target sites of sampling. Subgingival plaque (ca. 1.5 mg) from periodontal pockets (mean probing depths: 6.9 ± 2.1 mm; range: 4–10 mm) was pooled using sterile curettes and stored in a 1.5-mL sampling tube. Samples were transported on ice to the laboratory, and stored at -20°C pending analysis.

DNA extraction. Genomic DNA was extracted from

dental plaque using the InstaGene Matrix Kit (300 μ L, Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. The extracts were stored at -20°C .

PCR-dipstick DNA chromatography. Single-target PCR and dipstick DNA chromatography were performed in strict accordance with our previously reported protocol (35). All samples were individually analyzed for their content of 5 targeted bacterial species (*S. mutans*, *S. sobrinus*, *S. wiggisiae*, *Actinomyces* sp., and *V. parvula*). Species-specific primer sets were pre-labeled by tagging of each forward primer with one of five different oligonucleotides, and of all of the reverse primers with biotin, as reported previously (35). PCR amplification was performed with a mixture comprising 21 μ L of either plaque sample DNA or bacterial genomic DNA, 25 μ L of Taq DNA polymerase (HotStar Taq PLUS Master Mix; Qiagen GmbH, Hilden, Germany), and 4 μ L of primer mixture (5 μ M each) in a PCR Thermal Cycler MP (TaKaRa Biomedicals, Ohtsu, Shiga, Japan) or an iCycler (Bio-Rad Laboratories) programmed for one cycle of 5 min at 95°C (initial denaturation); 30 cycles of 1 min at 95°C for denaturation, 1 min at appropriate temperature for annealing (35), and 1.5 min at 72°C for extension; and a final extension cycle of 10 min at 72°C .

Dipstick strips (2.5 mm $\times$ 45 mm) were manufactured by immobilizing the respective complementary oligonucleotides in the test area to form 5 test lines (0.5 mm width; 1.2 mm apart from each other; Fig. 1). A control line (0.5 mm width) was formed at the end of each strip by applying biotin alone (35). Post-PCR analysis was carried out by diluting 1 μ L of each PCR amplification mixture with deionized water to a total volume of 30 μ L; combining the diluted mixture with 10 μ L of eluent (containing detergent, blocking agents, PBS and salts solution) and 2 μ L of streptavidin-coated blue latex suspension to prepare a chromatography solution; and then immersing the strip's wicking pad into the resulting solution. The strips and reagents were obtained commercially (TBA Co., Sendai, Japan). Tagged DNA amplicons were attached to blue latex microspheres through biotin-streptavidin interaction, migrated along the strip by capillary action, hybridized through molecular recognition of oligonucleotides to capture probes, and visualized as blue lines.

Semiquantification assay. Each PCR product was subjected to 10-fold serial dilution to maximum dilutions of 10^4 . One-microliter aliquot of the dilution

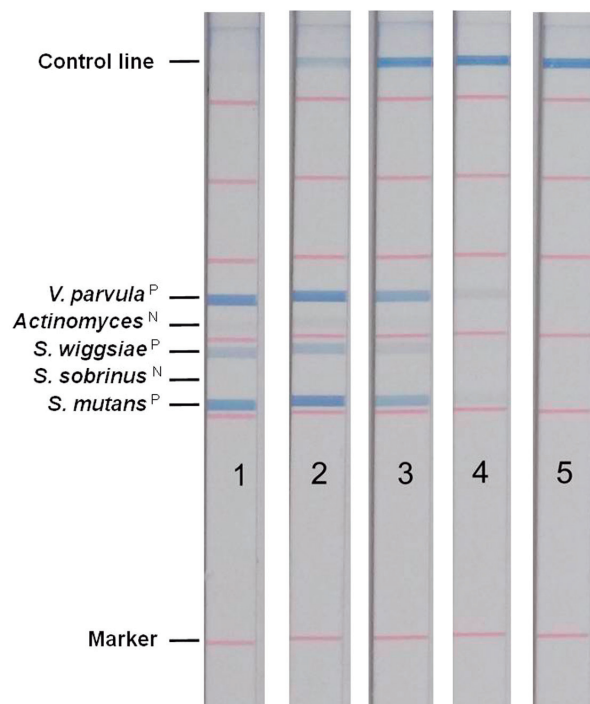


Fig. 1 Semi-quantitative chromatogram for sample 7. An aliquot (1 μ L) of each specific PCR amplification was assayed. Strips were ordered by increasing dilution factor (from 1–5, respectively): original concentration, 10-fold, 10^2 -fold, 10^3 -fold and 10^4 -fold. ^P: band detected from *Veillonella parvula*, *Scardovia wiggisiae*, and *Streptococcus mutans*; ^N: band not detected from *Actinomyces* sp. and *Streptococcus sobrinus*.

series of each PCR product (*i.e.*, five dipstick strips arranged by increasing dilution) from each plaque sample was assayed via semi-quantitative chromatography. The detection limit for each bacterial species was determined by the last visible band, and the corresponding titer (dilution fold, **D**) was recorded.

Data analysis. To estimate the relative concentration (C_r) of each bacterial species in a certain amount of dental plaque, the following equation was employed:

$$C_r = D / E_r$$

Where E_r represents the relative PCR amplification efficiencies for *S. mutans*, *S. sobrinus*, *S. wiggisiae*, *Actinomyces* sp., and *V. parvula*, which were estimated to be 1, 10^{-4} , 10^{-3} , 10^{-3} , and 10^{-2} , respectively (35). Detection limit observed at the original concentration (a single band in a chromatogram) was assigned a fold dilution of 1. Failure to detect a signal was recorded as zero.

Statistical analyses were performed between supra- and subgingival groups by SPSS. Independent *t*-test,

Table 1 Positively detected species (*S. mutans*, *S. sobrinus*, *S. wiggsiae*, *Actinomyces* sp., and *V. parvula*) in supra- and subgingival plaque samples

Characteristic	Supragingival plaque ^a	Subgingival plaque
Number of samples	16	16
Age (yr)	32 ± 9	56 ± 8
Female	9 (56.3%)	8 (50%)
Positively detected species in each sample*	3.2 ± 0.8	2.4 ± 0.7
Samples detected with ≥ 3 species	14	8
No. of samples with positively detected species		
1 species	0	2
2 species	2	6
3 species	10	8
4 species	3	0
5 species	1	0

Values are presented as mean ± SD or number (%)

^a Data derived from previous article (35).

* $P < 0.05$, independent *t*-test.

Fisher's exact probability test, and Mann-Whitney test were employed to determine a statistical difference of positively detected species, the detection frequencies of each bacterial species, and the relative amount of each bacterial species, respectively. $P < 0.05$ was considered statistically significant.

RESULTS

Qualitative analyses, including demographic characteristics, positively detected species and detection frequencies of each bacterial species, are summarized in Table 1 and Fig. 2. Data for supragingival groups are as presented in our preliminary report (35). Supragingival plaque (ca. 1.5 mg) from caries-free enamel surfaces was obtained from 16 healthy subjects (mean age, 31.8 ± 8.9 years; range, 23–54 years). Supragingival plaque harbored significantly more (mean 3.2) of the tested species (Table 1) and had a higher detection frequency for *V. parvula* than subgingival plaque (Fig. 2). Positively identified targets in supragingival plaque were 2 to 5 per sample and in subgingival plaque were 1 to 3. Samples determined to harbor 3 target species reached an overwhelming majority in both the supra- and subgingival groups. Notably, 4 out of 16 supragingival samples (25%) were positive for more than 3 of the selected bacteria, whereas no subgingival sample was.

Fig. 2 compares the detection frequencies of each tested species in supra- and subgingival groups. *V. parvula* exhibited a significantly reduced detection frequency in supragingival (93.8%) plaque compared to that in subgingival plaque (18.8%) ($P < 0.05$). In both supra- and subgingival plaque samples, *S. mutans* and *Actinomyces* sp. were highly prevalent,

whereas *S. sobrinus* and *S. wiggsiae* occurred at markedly lower frequencies. *S. sobrinus* was identified more frequently in subgingival plaque samples. Conversely, *S. wiggsiae* was better presented in supragingival plaque samples. However, neither of these differences achieved statistical significance ($P > 0.05$).

Fig. 3 displays the detection status and relative abundance of each tested species in plaque samples obtained from healthy coronal tooth surfaces and diseased periodontal pockets by dipstick chromatograms. For convenience, we assigned 7 levels to the relative concentrations and rearranged the samples in descending abundance to facilitate comparisons between the two groups. Thus, the plots with the same abscissa do not necessarily represent the same sample. The highest concentration (10^6) was estimated only with *Actinomyces* sp., which dominated over the other four species in supragingival plaque; these high levels of *Actinomyces* sp. were detected in all 16 samples. In subgingival plaque, *Actinomyces* sp. demonstrated similar predominance, albeit at significantly reduced abundance ($P < 0.05$, Mann-Whitney U test). Although half (8 of 16) of the subjects harbored subgingival *Actinomyces* sp. at the high level of 10^5 , a minority (2 of 16) of the subjects lacked bacteria of this type. The colonization of *V. parvula* in supragingival plaque differed in relative amount as well as in frequency from subgingival plaque, which was confirmed by Mann-Whitney U test ($P < 0.05$); *V. parvula* spanned a greater range of relative abundance (from 10 to 10^5) in supragingival plaque, while exhibiting low occurrence in subgingival plaque. *S. mutans* was present with less varied abundance in both groups. Among the 15 positive supragingival samples, *S. mutans* was de-

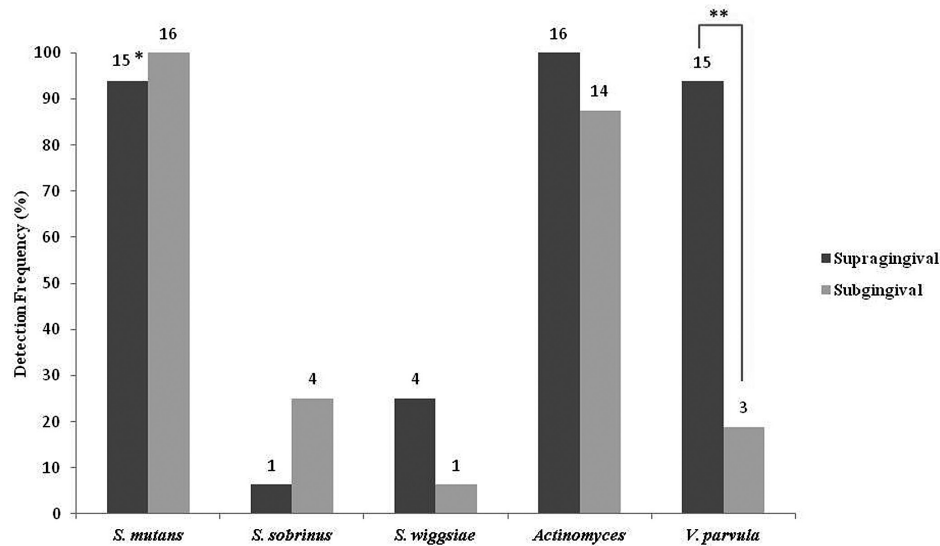


Fig. 2 Detection frequencies of *S. mutans*, *S. sobrinus*, *S. wiggsiae*, *Actinomyces* sp. and *V. parvula* in supra- and subgingival plaque samples. * Count of samples positively detected with each target species (n = 16). ** $P < 0.05$ by Fisher's exact probability test.

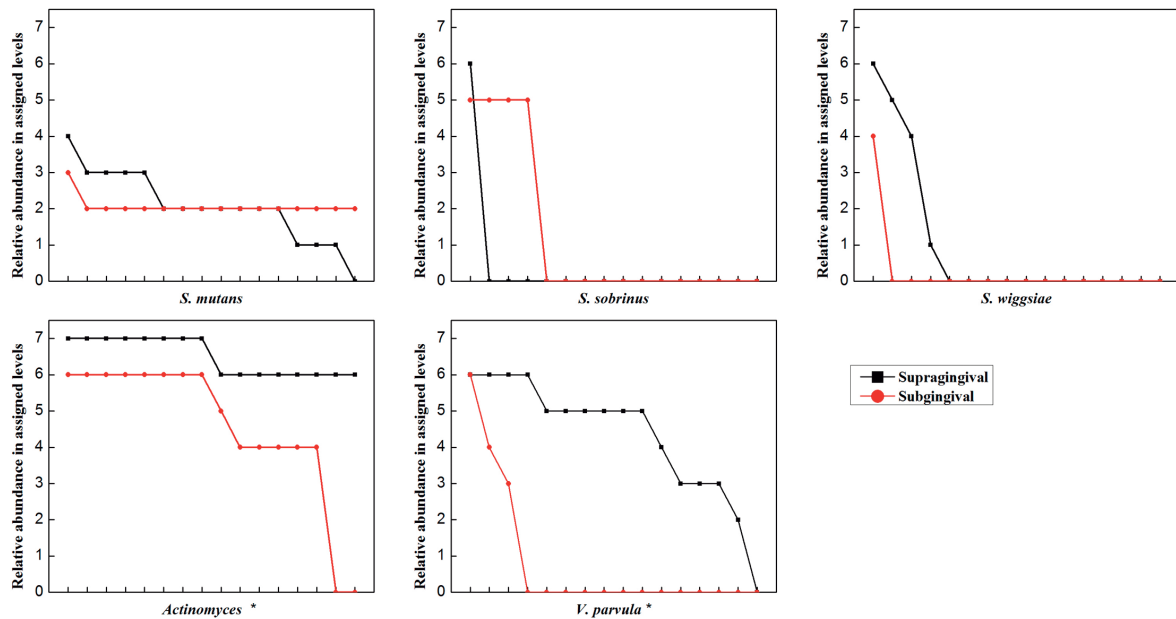


Fig. 3 Relative abundance of *S. mutans*, *S. sobrinus*, *S. wiggsiae*, *Actinomyces* sp., and *V. parvula* in supra- and subgingival plaque samples. Relative concentrations were assigned with scores from 1–7 as: 1, 1; 2, 10^1 ; 3, 10^2 ; 4, 10^3 ; 5, 10^4 ; 6, 10^5 ; 7, 10^6 . Not detected was recorded as zero. Samples were ordered in descending abundance of each species for supra- and subgingival groups. * $P < 0.05$ by Mann-Whitney U test comparison between supra- and subgingival groups.

tected at similar low levels in all except one sample (in which the level reached 10^3); similarly, *S. mutans* occurred at a concentration of 10 in all subgingival subjects except one (in which the level reached 10^2). In contrast, the other two frequently absent bacterial species of *S. sobrinus* and *S. wiggsiae* tended

to be highly abundant in positive samples.

DISCUSSION

In the present study, we demonstrated that PCR-dipstick DNA chromatography was sufficient for char-

acterizing a five-membered subset bacterial profile, and furthermore permitted comparison of the profiles among samples. Notably, these profiles allowed a rapid understanding of microbial community membership and structure at a glance of the chromatogram for each specimen.

Qualitative detection performed with a single strip was reliable for the study of community membership in terms of the combined sensitivity (from as low as 1 fg to 10 pg of extracted DNA) and exceptional specificity of PCR-dipstick DNA chromatography (35). The positive and negative tests analyzed both the occurrence of a test species in a single case and the prevalence of that species in a plaque pool. Plaque from healthy coronal surfaces harbored an average number of 3.2 of the target species per sample, a value significantly higher than that (2.4 species per sample) in plaque from diseased periodontal pockets, suggesting that the microbial community of the tooth surface had a more diverse cariogenic microflora. This result was not surprising when taking into account the physical heterogeneity of habitats above and below the gingival margin, *i.e.*, the site-specificity of microflora (2, 4, 17), along with the health or disease state of a habitat (37, 38). *V. parvula* exhibited a supragingival prevalence quintuple that observed subgingivally (93.8 versus 18.8%). While *V. parvula* appeared to be ubiquitous in intraoral sites (17, 38), this species lost the dominant role in periodontally diseased plaque. This result corresponds well with previous investigations that suggested *V. parvula* as a periodontal health-associated species (14, 15, 29). *S. mutans*, commonly taken as a caries initiator and a typical supragingival plaque resident, was (as expected) detected in the vast majority (93.8%) of the supragingival plaque pools, although this frequency was higher than that detected in previous reports (1, 2, 10, 25). However, unique to the present investigation was the subgingival prevalence of 100% for *S. mutans* in periodontal pockets. Despite the fact that many bacterial species are shared among different habitats in the human mouth, such a prevalence was still considerably higher than that observed in previous reports (7, 23, 24, 34). These ambiguities probably arise from differences in the sensitivity of selected detection strategies, as well as differences in sampling sites, time and methods in various reports. The preference of *Actinomyces* sp. for colonization in supra- and subgingival plaque (2, 17), and the low occurrence of *S. sobrinus* and *S. wiggisiae*, were well corroborated in this study (25, 33). *S. sobrinus* and *S. wiggisiae* did not differ significantly in prevalence between supra- and subgingival

gingival plaque. However, *S. sobrinus* appeared to occur more frequently in subgingival plaque.

When it comes to either predicting or diagnosing a plaque-mediated disease, the richness of putative pathogens is considered far more important than the presence or absence. For instance, the onset of dental caries is often observed with elevated levels of acidogenic and aciduric bacteria (18, 31), and the onset of periodontal disease is often correlated with elevated levels of “red” and “orange” complex species (27). In the present study (as shown in Fig. 2), plaque from diseased periodontal pockets exhibited an overall decrease in relative abundance of detected species compared to that of plaque from healthy tooth surfaces. The reason could be that the five species examined here have long been thought to be cariogenic; a decrease in the abundance of these 5 species might occur in compensation for an increase in the abundance of putative periodontal pathogens (16, 37, 38). This investigation once again confirmed the supragingival and subgingival predominance of *Actinomyces* sp. within the five-membered bacterial profile (37, 38). *V. parvula*, together with *Actinomyces* sp., exhibited significantly reduced abundance in periodontal pockets compared to tooth surfaces. In the context of another observation—specifically, that *S. mutans* maintained in low levels throughout all subgingival specimens—this parallel reduction was not surprising, given that *V. parvula* can use lactate as a sole nutrient source and that *V. parvula* is known to exhibit a coactive relationship with acidogenic bacteria such as *S. mutans* and *Actinomyces* sp. (20, 26).

The unique advantage of PCR-dipstick DNA chromatography is that the semiquantitative analysis enabled discrimination between bacterial profiles at the species level. This aspect permitted us to demonstrate that the predominant subset profile on healthy coronal surfaces was predominated by *Actinomyces* sp. with lower abundances of *V. parvula* and *S. mutans*, while the profile lacked *S. sobrinus* and *S. wiggisiae*. In contrast, in periodontal pocket the predominance of any one profile was obscured where several overlapped profiles were detected at comparable frequencies. One profile (consisting primarily of *Actinomyces* sp. with lower levels of *S. mutans* while lacking *V. parvula*, *S. sobrinus*, and *S. wiggisiae*) was found in the largest number of specimens. Notably, in another frequently detected subgingival profile, *S. sobrinus* dominated in *S. sobrinus*-positive plaque, with a concomitant decrease in *Actinomyces* sp. abundance, suggesting a competitive rather than cooperative relationship between *S. sobrinus* and *Actinomyces* sp. In addition, comparison

between individual subjects revealed interindividual variations of profiles, as shown in Fig. 3. Because specimens from tooth surfaces and periodontal pockets were pooled, the interindividual difference might be somewhat overestimated without rigorously controlling for general oral health and disease states (e.g., healthy enamel of healthy or diseased oral cavity) (1), or sampling sites (e.g., different depths of periodontal pockets) (27). Moreover, since the number of subjects in this study was relatively small, we expect the interindividual differences would be minimized and the predominant profiles in different sample collections might become apparent when larger numbers of subjects are recruited.

The present PCR-dipstick DNA chromatography enabled simultaneous profiling of multiple species (currently, up to 12 on a single strip) within the same plaque sample, and facilitated discrimination between anticipated different microflora, which would not have been possible by assaying with previous strip biosensors. The exquisite sensitivity of DNA biosensors, combined with PCR amplification, set the detection threshold at a low level. The semi-quantitative protocol was established to meet the requirement for samples to be processed quickly at chair-side, permitting the use of miniaturized apparatus in a user-friendly format. Theoretically, a single strip could identify the community membership of a subgroup of bacteria and a maximum of five strips would be sufficient for analyzing its member structure. However, we are aware that the present assay is not without its limitations. It should be noted that this assay provides only relative abundance, which does not necessarily reflect the actual amount of a bacterial species. Additionally, PCR-dipstick DNA chromatography only permitted the analysis of a finite group of already characterized species, since this method is not an open-ended technique and relies on PCR amplification with species-specific primers. An anticipated concern is that testing only a small subgroup of bacteria could cause an oversimplification of the actual microflora; indeed the defined bacterial species may not be directly involved in the etiology of a disease. As already discussed, the bacterial flora are expected to undergo a successional change in composition during disease progression, as evidenced by increased levels of putative pathogens along with decreased levels of innocent bystanders. Those species that exhibit a typical change in abundance might be selected as candidates to characterize the microbiota. Nonetheless, a panel of specific pathogens would be ideal. In future studies, additional bacterial species (including those that are

thought to be associated with endodontic and periodontal infection) should be incorporated in the further evaluation of this assay. Moreover, characteristic subgroups of bacterial species should be identified to serve as markers of healthy or diseased tissue.

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