

Comparison between polymerase chain reaction-based and checkerboard DNA hybridization techniques for microbial assessment of subgingival plaque samples

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Abstract

Aim: To compare polymerase chain reaction (PCR) with subsequent reverse hybridization (micro-IDent test) and checkerboard DNA–DNA hybridization for the identification of 13 bacterial species in subgingival plaque samples.

Material and Methods: Subgingival plaque samples were taken using paper points and curettes from two sites each with pocket depth <4, 4–6 and >6 mm at baseline and 3 months in 25 periodontitis subjects and two sites in 25 periodontally healthy subjects. Samples were analysed for their content of 13 bacterial species using both assays. Similarities for each species between techniques were determined using regression analysis. Differences between health and periodontitis were determined using the Mann–Whitney test.

Results: Three hundred and fifty samples were evaluated using both techniques. Regression analysis indicated that 10/13 test species showed significant positive correlations between the counts determined by checkerboard analysis and levels determined by the PCR-based test after adjusting for 13 comparisons. The highest rank correlations of 0.58, 0.49 and 0.46 were seen for *Treponema denticola*, *Fusobacterium nucleatum* and *Eubacterium nodatum*, respectively ($p < 0.0001$). Both tests could distinguish samples from healthy and periodontitis subjects.

Conclusion: Detection patterns of 10/13 test species in subgingival plaque samples from periodontitis and healthy subjects were similar using the two molecular techniques.

Key words: bacteria; checkerboard; DNA probes; PCR; subgingival biofilm

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Periodontal diseases are infections caused primarily by bacteria living in

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plaque biofilms. Therefore, the detection of specific bacterial species in subgingival biofilm samples could aid in determining an individual's risk of disease, the nature of an optimal periodontal therapy as well as microbial outcomes post-therapy. A limited number of periodontal diagnostic tests have been available to the clinician for many

years, including culture, whereby patient samples are sent to a laboratory for enumeration of bacterial species using cultural techniques. Chair-side tests have also been available such as the BANA test that detects the small number of species that can hydrolyse benzoyl-DL-arginine-naphthylamide including the periodontal pathogens

Porphyromonas gingivalis, *Tannerella forsythia* and *Treponema denticola* (Loesche et al. 1992).

The detection and identification of bacterial species in subgingival plaque samples using cultural techniques is an expensive and labour-intensive procedure and limits the number of samples that can be evaluated. The advent of molecular techniques has facilitated examination of the microbial composition of subgingival plaque samples. Some of the molecular techniques such as conventional polymerase chain reaction (PCR) provide presence or absence of data, others such as the checkerboard DNA–DNA hybridization method or RT-PCR provide quantitative data, while still others such as PCR hybridization provide semi-quantitative data. What is not clear is how the results of techniques such as PCR compare with other microbial identification techniques. PCR was compared with culture for the detection of *Aggregatibacter actinomycetemcomitans* and *P. gingivalis* in 173 plaque samples from 43 subjects (Riggio et al. 1996). PCR demonstrated a higher frequency of detection of the two periodontal pathogens than culture. Siqueira et al. (2002) compared 16S rDNA-based PCR and the checkerboard technique for the content of *A. actinomycetemcomitans*, *T. forsythia*, *Parvimonas micra*, *Porphyromonas endodontalis*, *P. gingivalis* and *T. denticola* in samples from root canals. They found that matching results ranged from 60% to 97.5% depending on the target species and that the major discrepancies occurred due to PCR-positive but checkerboard-negative results. RT-PCR has also been compared with cultural methods (Boutaga et al. 2003, 2005, 2006, 2007, Lau et al. 2004, Jervoe-Storm et al. 2005), again with decent agreement and a modest increase in sensitivity of detection with RT-PCR.

Molecular techniques have been developed into diagnostic tests for use by clinicians. The micro-IDent test employs PCR, to amplify the sample DNA, and specific DNA probes on a DNA-STRIP® (Hain Lifescience GmbH, Nehren, Germany) matrix to hybridize with the amplified DNA. This reaction provides a colour reaction on the DNA-STRIP®, the intensity of which corresponds to the initial level of DNA in the sample. Eick & Pfister (2002) compared the micro-Dent(R) kit (Hain Lifescience GmbH) with culture (micro-

scopy for spirochetes) for the detection of *A. actinomycetemcomitans*, *P. gingivalis*, *Prevotella intermedia*, *T. forsythia* and *T. denticola* in 122 plaque samples from sites with different pocket depths (PD). Both techniques showed a positive correlation between PD and the quantity of the test species. However, the micro-Dent(R) kit identified *P. gingivalis* and *T. forsythia* more often than culture.

Since the study of Eick & Pfister (2002), the range of species identified by the micro-IDent test has been increased from 5 to 13. Further, this PCR-based test has not been compared with the checkerboard technique that has been employed in numerous oral ecological and periodontal treatment studies (Socransky & Haffajee 2005, Haffajee et al. 2006a, b). Thus, the purpose of the present investigation was to compare two molecular methods, PCR with subsequent reverse hybridization (micro-IDent test) and checkerboard DNA–DNA hybridization, for the identification of 13 bacterial species in subgingival plaque samples taken from subjects who were periodontally healthy or had evidence of periodontitis.

Material and Methods

Subject population and clinical monitoring

Twenty-five chronic periodontitis and 25 periodontally healthy subjects were recruited. Periodontally healthy subjects were >20 years of age, had at least 24 teeth and no sites with PD or attachment level (AL) ≥ 4 mm. Periodontitis subjects were >20 years of age, had at least 20 teeth and >5% of sites with PD ≥ 4 mm and/or >5% of sites with AL ≥ 4 mm. All subjects were in good general health and had not received periodontal treatment or systemic antibiotics in the previous 3 months. The study was approved by The Forsyth Institute Institutional Review Board and conformed to the guidelines in the Declaration of Helsinki. All subjects provided signed informed consent before entry into the study.

Subjects were measured for periodontal status using the following clinical measurements: plaque accumulation (0/1), overt gingivitis (0/1), bleeding on probing (0/1), probing PD and probing AL measured at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) at all teeth excluding third molars.

Microbiological assessment

Subgingival plaque samples were taken from six sites in the periodontitis subjects and included two each with PD <4, 4–6 and >6 mm. The same sites were re-sampled at 3 months. Sites were sampled first using two paper points (for PCR) followed by a curette sample for the checkerboard technique. Two healthy sites were sampled in the periodontally healthy subjects using paper points and curettes at one visit only. The different methods of sampling were employed in order to compare the microbiological findings from the sampled sites using the actual sample collection method employed for the micro-IDent and checkerboard techniques.

For the micro-IDent method, the paper points were inserted into the sulcus/pocket of the test teeth and left in place for 20 s. They were then placed into individual 1.5 ml screw capped tubes, to which 200 μ l of 5% Chelex solution, a chelating cation resin suspension used for the rapid extraction of DNA from small biological samples, was added. Tubes were centrifuged, and then disrupted in a heated sonic water bath. The samples were vortexed then heated at 105°C. After centrifugation, 5 μ l of supernatant was used for PCR. The samples were amplified by multiplex PCR using *taq* polymerase enzyme and biotinylated primers. Negative and positive amplification controls were included in each run. The amplified product was chemically denatured and the single-stranded biotin-labelled amplicons resulting from the PCR step were hybridized to membrane-bound species-specific probes. The membranes were stringently washed and then the signals detected by addition of a streptavidin–alkaline phosphatase conjugate followed by an alkaline phosphatase-mediated staining reaction. The signals on the membrane were visually compared with a template provided by the manufacturer and scored as 0.5, 1, 2 and 3 depending on the intensity of the signal.

The curette samples for the checkerboard technique were placed in individual Eppendorf tubes (VWR, West Chester, PA, USA) containing 0.15 ml Tris EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). 0.10 ml of 0.5 M NaOH was added immediately to each sample. Each sample was evaluated for its content of 13 bacterial species using checkerboard DNA–DNA hybridization (Socransky et al. 1994, 2004). In brief, the samples were

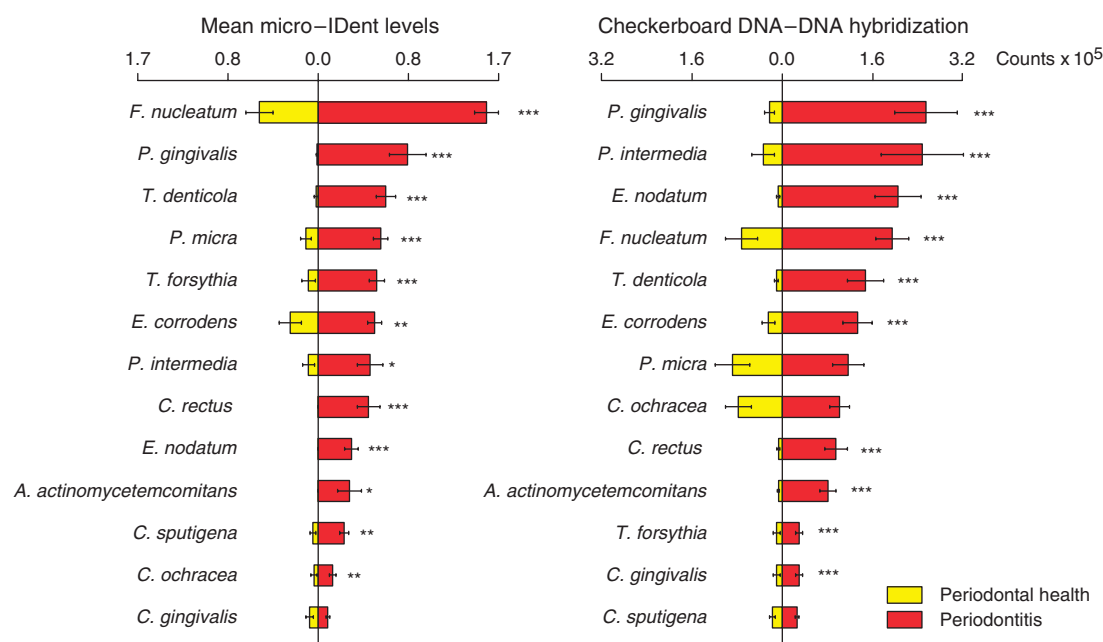


Fig. 1. Mean levels (\pm SEM) of 13 test species in 25 periodontally healthy and 25 periodontitis subjects. Counts or levels were averaged within each subject and then averaged across subjects in the two clinical groups separately. Significance of differences for each species was sought using the Mann-Whitney test and adjusted for 13 comparisons (Socransky et al. 1991). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Species were ordered separately in each panel according to levels/counts in the periodontitis subjects.

lysed and the DNA placed in lanes on a nylon membrane using a Minislot device (Immunetics, Cambridge, MA, USA). After fixation of the DNA to the membrane, the membrane was placed in a Miniblotter 45 (Immunetics), with the lanes of DNA at 90° to the lanes of the device. Digoxigenin-labelled whole genomic DNA probes to 40 bacterial taxa were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes detected using antibody to digoxigenin, conjugated with alkaline phosphatase and chemifluorescence detection. Signals were detected using AttoPhos substrate (Amersham Life Sciences, Arlington Heights, IL, USA) and were read using a Storm FluorImager (Molecular Dynamics, Sunnyvale, CA, USA), a computer-linked instrument that reads the intensity of the fluorescence signals resulting from the probe-target hybridization. Two lanes in each run contained standards at the concentration of 10⁵ and 10⁶ cells of each species. The sensitivity of the assay was adjusted to permit the detection of 10⁴ cells of a given species by adjusting the concentration of each DNA probe. Signals were evaluated using the Storm FluorImager and converted to absolute counts by comparison

Table 1. Overall agreement, sensitivity and specificity of the micro-IDent method using checkerboard DNA-DNA hybridization counts $> 10^5$ as the "gold standard"

Species	Threshold for micro-IDent	% Agreement	Sensitivity	Specificity
<i>Porphyromonas gingivalis</i>	0.5	82.9	77.0	84.6
<i>Treponema denticola</i>	0.5	78.7	76.4	79.3
<i>Fusobacterium nucleatum</i>	0.5	64.2	93.7	47.3
<i>Eubacterium nodatum</i>	0	78.7	56.0	86.5
<i>Campylobacter rectus</i>	0	77.1	64.8	79.4
<i>Capnocytophaga ochracea</i>	0	74.2	47.9	80.5
<i>Parvimonas micra</i>	0	54.2	80.0	45.2
<i>Eikenella corrodens</i>	0	58.1	69.7	54.9
<i>Aggregatibacter actinomycetemcomitans</i>	0	80.4	23.4	89.6
<i>Prevotella intermedia</i>	0	70.8	38.5	78.8
<i>Tannerella forsythia</i>	0	61.5	56.3	61.8
<i>Capnocytophaga sputigena</i>	0	70.1	42.9	71.3
<i>Capnocytophaga gingivalis</i>	0	82.2	25.0	85.0
Overall mean (\pm SD)		71.8 \pm 9.6	57.8 \pm 21.7	72.6 \pm 15.3
Overall median (95% CI)		74.2 (61.5–80.4)	56.3 (38.5–77.0)	79.3 (54.9–85.0)

The threshold for the micro-IDent was that giving the highest χ^2 value for each species.

with standards on the same membrane. Failure to detect a signal was recorded as zero. The species evaluated by both methods were *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *T. denticola*, *P. intermedia*, *P. micra*, *Fusobacterium nucleatum*, *Campylobacter rectus*, *Eubacterium nodatum*, *Eikenella corrodens*, *Capnocytophaga gingivalis*, *Capnocyto-*

phaga ochracea and *Capnocytophaga sputigena*. A total of 350 samples were analysed by each method.

Data analysis

Similarity between samples evaluated using the two techniques was determined using regression analysis. Differ-

ences between healthy and periodontitis subjects and among PD categories were determined using the Mann–Whitney and Kruskal–Wallis tests and adjusted for 13 comparisons (Socransky et al. 1991).

Results

Both methods easily distinguished samples from periodontally healthy individuals from samples from periodontitis subjects (Fig. 1). Twelve of 13 species evaluated using the micro-IDent test were significantly different between periodontal health and disease after adjusting for 13 comparisons (Fig. 1, left panel). Ten of 13 species differed significantly using checkerboard DNA–DNA hybridization (Fig. 1, right panel). Many species in samples from different pocket ranges also differed significantly by both techniques (data not shown).

The next question asked was whether the checkerboard DNA–DNA hybridization technique and the micro-IDent technique detected (or failed to detect) the test species in samples from the same sites. Table 1 presents the overall agreement, sensitivity and specificity of each test species of the micro-IDent technique using the detection of a species at $>10^5$ by the checkerboard technique as the ‘gold standard’. The threshold of 10^5 was chosen based on the findings that the odds of sites harbouring periodontal pathogens greater than this threshold is far higher in subgingival plaque samples from periodontally diseased than healthy subjects (Haffajee et al. 2006a, b) and this threshold was related to the risk for periodontal disease progression (Haffajee et al. 1991). The threshold for the micro-IDent technique was chosen by seeking the highest χ^2 value for each species when compared with the checkerboard value of $>10^5$ cells. The median per cent agreement, sensitivity and specificity for all the 13 test species were 74.2, 56.3 and 79.3, respectively.

Because both the micro-IDent and checkerboard methods provided data on at least an interval scale, the relationship between micro-IDent categories and species counts using checkerboard DNA–DNA hybridization was examined using the Spearman rank correlation coefficient (Table 2). The Spearman rank correlation coefficients exceeded 0.270 ($p < 0.001$) for nine of 13 comparisons including *T. denticola*,

F. nucleatum, *E. nodatum*, *C. rectus*, *P. gingivalis*, *P. micra*, *C. ochracea*, *T. forsythia* and *E. corrodens*. The four remaining comparisons, *A. actinomycetemcomitans*, *P. intermedia*, *C. sputigena* and *C. gingivalis* were positive, but not statistically significant after adjusting for 13 comparisons.

The data were explored further in Fig. 2 which plots the levels of *P. gingivalis* as determined by the micro-IDent system (x-axis) against the

counts of *P. gingivalis* determined using checkerboard DNA–DNA hybridization (y-axis). A Tukey box plot was superimposed on the columns of the micro-IDent values. The regression line in Fig. 2 demonstrated the strong positive correlation between the levels of *P. gingivalis* as determined by the two techniques. The regression line passes approximately through zero indicating that there were large numbers of zero values by both the micro-IDent and

Table 2. Relationship between micro-IDent category of individual species and counts of the same determined using checkerboard DNA–DNA hybridization

Species	Spearman rank correlation coefficient	<i>p</i>
<i>Treponema denticola</i>	0.577	0.00000
<i>Fusobacterium nucleatum</i>	0.490	0.00000
<i>Eubacterium nodatum</i>	0.459	0.00000
<i>Campylobacter rectus</i>	0.444	0.00000
<i>Porphyromonas gingivalis</i>	0.443	0.00000
<i>Parvimonas micra</i>	0.413	0.00000
<i>Capnocytophaga ochracea</i>	0.293	0.00000
<i>Tannerella forsythia</i>	0.289	0.00000
<i>Eikenella corrodens</i>	0.273	0.00001
<i>Aggregatibacter actinomycetemcomitans</i>	0.167	0.00215
<i>Prevotella intermedia</i>	0.154	0.00426
<i>Capnocytophaga sputigena</i>	0.154	0.00434
<i>Capnocytophaga gingivalis</i>	0.145	0.00695

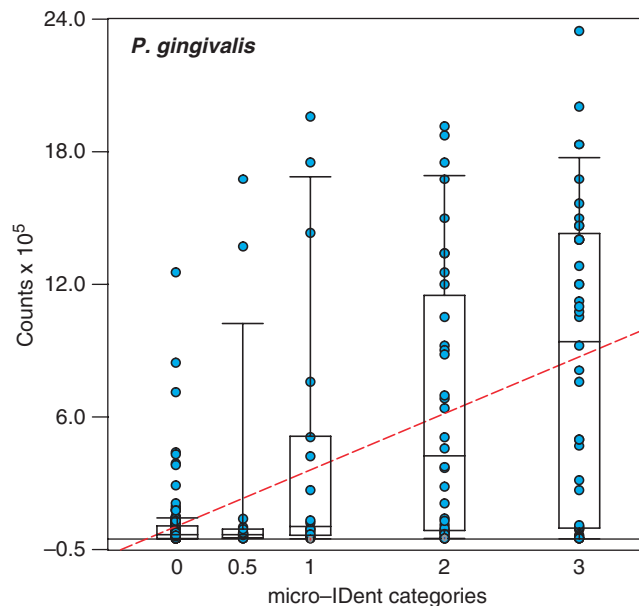


Fig. 2. Scatter plot and Tukey box plot of the relationship of *Porphyromonas gingivalis* levels in subgingival plaque samples determined by the micro-IDent system and checkerboard DNA–DNA hybridization. The x-axis represents the micro-IDent levels while the y-axis represents the counts of *P. gingivalis* $\times 10^5$ as determined by checkerboard DNA–DNA hybridization. Because there were five discrete micro-IDent values, it was possible to superimpose a Tukey box of the checkerboard DNA–DNA hybridization values on each of the five columns of values. For the Tukey box plots, the rectangular box represents the upper and lower quartiles, the horizontal line in each box the median value, and the whiskers the upper and lower 90% levels. The dotted red line represents the regression line relating the micro-IDent and checkerboard levels of *P. gingivalis*.

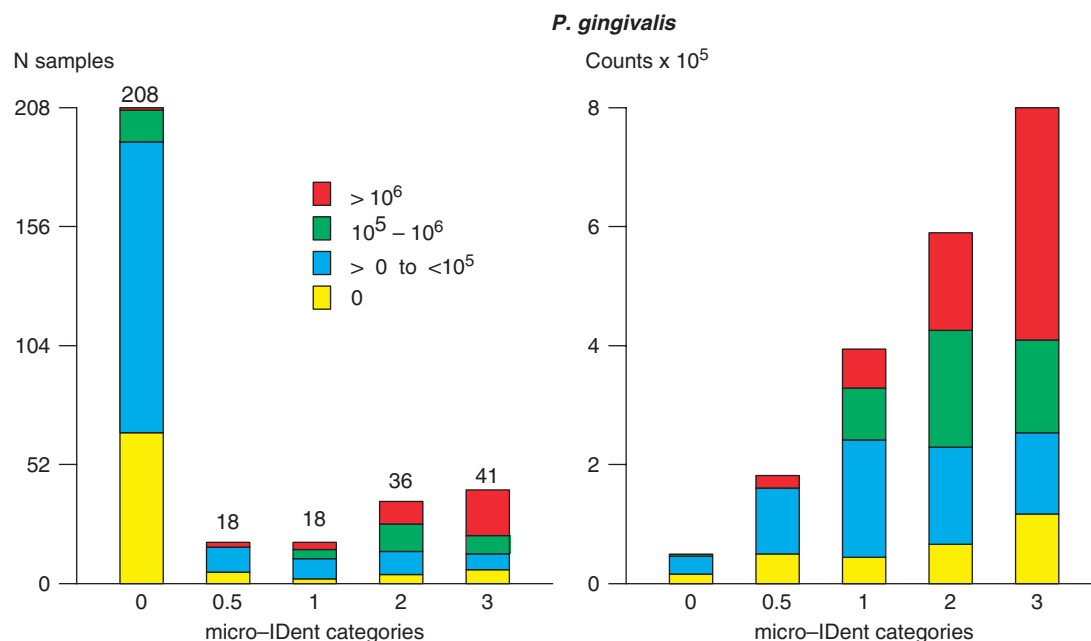


Fig. 3. Number of samples in count categories for data subset according to micro-IDent levels of *Porphyromonas gingivalis*. Left panel indicates the number of samples in each of the five micro-IDent categories that had checkerboard counts of *P. gingivalis* of 0, < 10⁵, 10⁵–10⁶ and > 10⁶. The right panel represents the mean checkerboard DNA probe counts for samples subset according to the micro-IDent categories. The coloured bars in each stack indicate the proportion of that stack represented by counts of 0, < 10⁵, 10⁵–10⁶ and > 10⁶.

checkerboard methods which are not clearly seen due to superimposition of the 0,0 data points. This is clarified in Fig. 3 (left panel) which indicates the numbers of samples in *P. gingivalis* count categories (determined by checkerboard DNA–DNA hybridization) of 0, < 10⁵, 10⁵–10⁶ and > 10⁶ for each of the micro-IDent categories. Most notable in Fig. 3 is that 208 samples were classified as having no *P. gingivalis* by the micro-IDent test. The vast majority of samples taken from these sites using curettes for analysis by checkerboard DNA–DNA hybridization had 0 or < 10⁵ *P. gingivalis* (blue and yellow areas of the bar). The right panel of Fig. 3 demonstrates that the mean counts of *P. gingivalis* increased with increasing micro-IDent level at the same sampled site. In addition, the proportion of high checkerboard counts increased with increasing micro-IDent levels. Similar findings were observed for other species such as *E. nodatum* (Figs 4 and 5). Figures 6 and 7 present similar data for *A. actinomycetemcomitans*. This species showed the worst sensitivity of detection using the micro-IDent system as well as a low correlation coefficient (Table 2). The left panel of Fig. 7 demonstrates the large number of zeros by the micro-IDent technique as well as the large number of samples in the zero micro-IDent category with

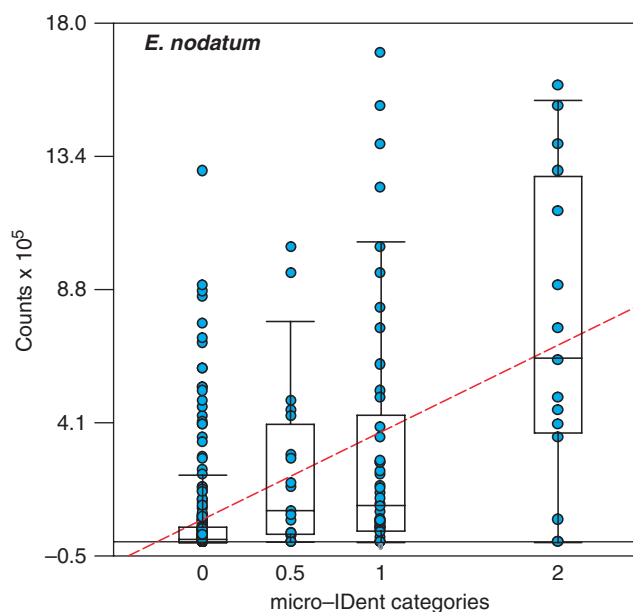


Fig. 4. Scatter plot and Tukey box plot of the relationship of *Eubacterium nodatum* levels in subgingival plaque samples determined by the micro-IDent system and checkerboard DNA–DNA hybridization. The layout of the figure is as described for Fig. 2.

counts of 0 or < 10⁵ determined by the checkerboard technique.

Discussion

The goal of this investigation was to compare the microbiological composi-

tion of subgingival biofilm samples using a PCR hybridization system (micro-IDent) and a direct DNA probe method (checkerboard hybridization). This is simple in concept, but more difficult to conduct. A study of this type necessitates comparison of the microbial composition of mixed bacter-

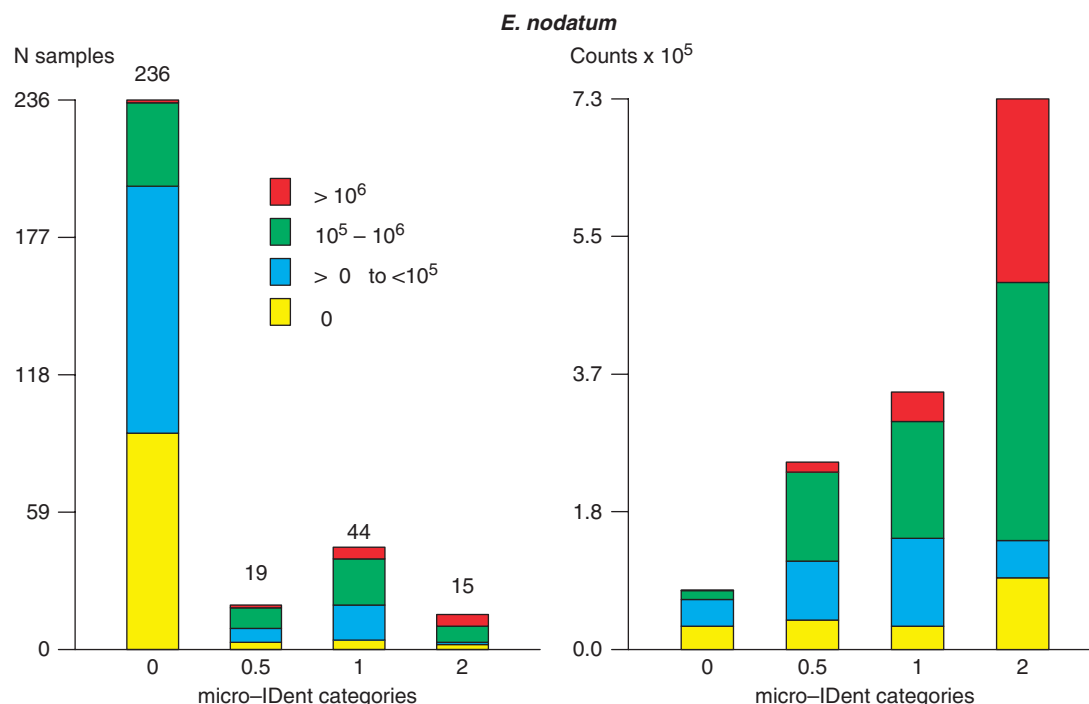


Fig. 5. Number of samples in count categories for data subset according to levels of *Eubacterium nodatum*. The layout of the figure is as described for Fig. 3.

ial ecosystems taken from subgingival sites. Subgingival biofilm communities are not homogeneous in the mixture of species and also species exist in micro-colonies that are not evenly distributed throughout the subgingival area. For this reason repeated sampling of a subgingival site even by the same sampling method and using the same enumeration technique will inevitably give similar but somewhat different results because the starting samples would be different (Teles et al. 2007). One method to overcome this difficulty might be to split the source sample. However, split samples are rarely of similar composition because bacterial cells in subgingival biofilms are often very adherent making the preparation of a homogeneous suspension extremely difficult. Thus, it would be unlikely that one could obtain essentially identical samples by either repeated sampling of the same subgingival site or by splitting a single sample. These difficulties were compounded by using different sampling methods, those recommended for each technique; i.e. paper points for the micro-IDent technique and curette samples for the checkerboard technique. It has been shown that paper point sampling gives different information than scaler/curette sampling of the same sites even when using the

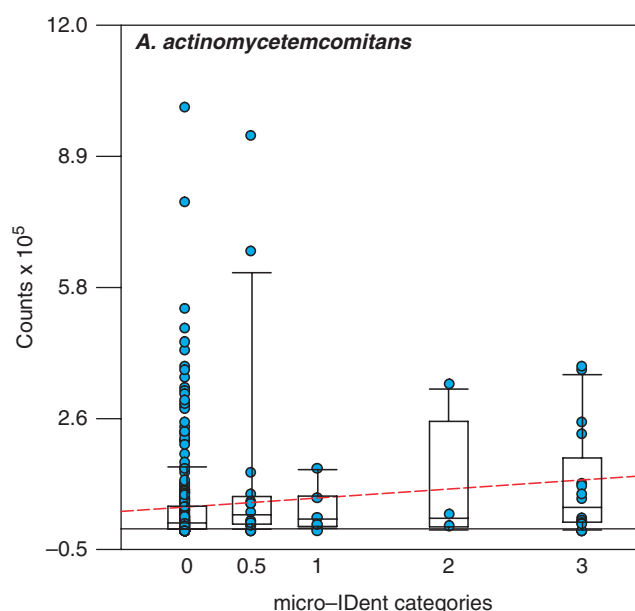


Fig. 6. Scatter plot and Tukey box plot of the relationship of *Aggregatibacter actinomycetemcomitans* levels in subgingival plaque samples determined by the micro-IDent system and checkerboard DNA–DNA hybridization. The layout of the figure is as described for Fig. 2.

same microbiological enumeration techniques (Tanner & Goodson 1986, Baker et al. 1991, Renvert et al. 1992).

Given the recognition that samples, even when taken from the same sub-

gingival site, will not have identical microbial composition and that adherent organisms in subgingival samples cannot be distributed uniformly into subsamples, the question remained as to the

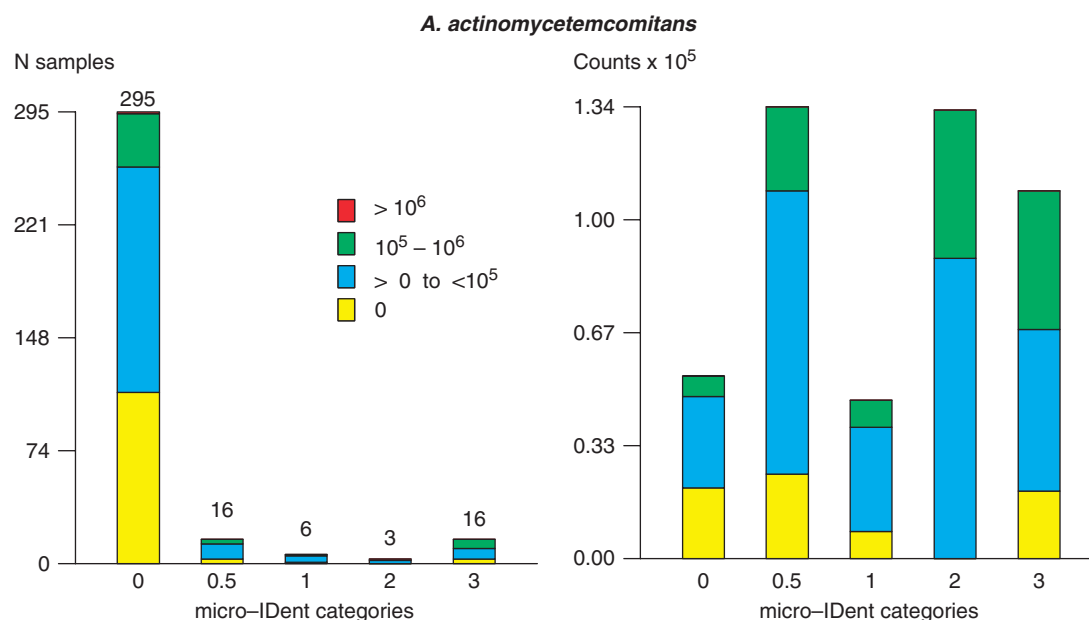


Fig. 7. Number of samples in count categories for data subset according to levels of *Aggregatibacter actinomycetemcomitans*. The layout of the figure is as described for Fig. 3.

criteria to be employed to determine whether two different microbiological assessments would provide comparable results. First, it was felt that when samples from a site gave high counts or levels by one method that the majority of time the other method should also give high counts of the same species. This comparison was carried out using regression analysis where high correlation coefficients and significance levels would indicate comparability. Second, it was felt that because many samples would provide zero levels on the micro-IDent scale or zero or low counts by checkerboard DNA–DNA hybridization, then concordance in zero values and low counts should usually be observed between the two methods for most species. Zero values and counts $< 10^5$ were combined for the checkerboard method since values below 10^5 typically were not considered to be associated with disease. Finally, it was felt that the two methods should show concordance in distinguishing subgingival samples from periodontal health from those obtained from periodontal disease.

The results of this study were quite remarkable given the constraints of this type of in vivo comparison. Concurrence, as determined by regression analysis, between the micro-IDent test and checkerboard DNA–DNA hybridization was very good for certain test species including *P. gingivalis*, *T. denticola*,

F. nucleatum, *E. nodatum*, *C. rectus*, *P. micra* and *C. ochracea* reasonably good for *T. forsythia*, *E. corrodens* and *A. actinomycetemcomitans*, while the relationship between the two assays for *C. sputigena*, *C. gingivalis* and *P. intermedia* was less strong. The results also indicated that many of the samples were negative for the test species as determined by the micro-IDent test while corresponding counts for the checkerboard technique were usually either 0 or $< 10^5$. Both techniques could readily distinguish between samples from periodontally healthy and periodontitis subjects as well as samples from different PD categories (data not shown).

While comparing different microbial enumeration techniques, it is unlikely that there will be perfect agreement (Riggio et al. 1996, Ali et al. 1997, Papapanou et al. 1997, Moraes et al. 2002, Siqueira et al. 2002, Boutaga et al. 2003, 2005, 2006, 2007, Smola et al. 2003, Lau et al. 2004, Jervoe-Storm et al. 2005). The general concordance of the two methods examined in the current investigation is important in that each provides validation for the other. This is reassuring because the selection of species for the micro-IDent system was based largely on data derived using the checkerboard DNA–DNA hybridization system. The availability of the two methods provides clinicians/investigators with a choice of microbial identification techniques

that they might employ to analyse oral biofilm samples.

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Clinical Relevance

Scientific rationale for the study: Multiple techniques are being used to identify bacterial species in oral biofilm samples. It is essential to determine whether different techniques provide similar results.

Principal findings: 10/13 test species showed significant positive correlations between the counts determined by checkerboard analysis and levels determined by the PCR-based test after adjusting for 13 comparisons.

Practical implications: These two methods, when used according to the stipulated protocol, provide similar results when used to evaluate the content of subgingival biofilm samples.

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