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A new checkerboard panel for testing bacterial markers in periodontal disease

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Background/aims: Various microbiological methods have been used for testing bacterial markers for periodontitis and periodontal disease progression. Most studies have used only a limited number of well recognized bacterial species. The purpose of the present study was to evaluate the association of 13 more recently identified bacterial species in a new panel in comparison with 12 previously more recognized periodontotopathogens ('old panel') using the 'checkerboard' DNA–DNA hybridization method.
Methods: Fifty individuals were chosen who showed at least one site with a probing pocket depth of 6 mm or more (disease) and bleeding on probing and at least one site with a probing pocket depth of 3 mm and without bleeding on probing (health). One diseased and one healthy site on each individual were sampled with the paperpoint technique and the samples were processed in the checkerboard technique against deoxigenin-labeled whole genomic probes to 25 subgingival species representing 12 well recognized and 13 newly identified periodontitis associated species.
Results: Twenty-four (out of 25) species were detected more frequently in the subgingival plaque of diseased than healthy sites both at score 1 ($> 10^4$) and score 3 ($> 10^5$). A significant difference at the higher score (score 3) was noticed for all species of the old panel except for three (*Streptococcus intermedius, Selenomonas noxia*, and *Eikenella corrodens*). Of the species in the new panel only *Prevotella tannerae, Filifactor alocis*, and *Porphyromonas endodontalis* showed a statistical significant difference between diseased and healthy sites.
Conclusion: It was concluded that *P. tannerae, F. alocis*, and *P. endodontalis* should be added to the 12 species used for routine diagnostics of periodontitis-associated bacterial flora.

Key words: bacterial markers; DNA probes; microbiology; periodontitis; subgingival plaque

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The subgingival flora of the deep periodontal pocket in periodontitis cases shows a quite different composition from that of the shallow pockets of nonperiodontitis cases (4, 11). A similar difference in the subgingival microbiota between deep and shallow pockets has also been shown within the same individual, although with less consistency (41). This difference has led to a specific interest to use microbiological sampling and analysis for diagnostic purposes, to provide guidance for treatment strategy and for evaluation of treatment (5, 16, 27, 37). The microflora associated with deep pockets has thus been interpreted as disease-associated and the presence of certain putative pathogens has been used to assess the risk for further periodontitis progression (7, 14, 19, 22, 23, 34, 38). *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis*, and *Tannerella forsythia* have been used as indicators of periodontal disease severity due to their association with deep periodontal pockets and periodontal progression (1). Even if other species have been included for microbiological diagnosis in periodontitis cases, their use in a clinical setting has been limited due to shortcomings of culture methods.

New molecular-based methods such as the 'checkerboard' DNA–DNA hybridization method have made it possible to evaluate the presence and to quantify up to 40 bacterial species in a large number

of plaque samples (30). A relation between periodontal progression has thus been attributed to certain combinations or 'complexes', e.g. red and orange complex, whereas others, e.g. green and yellow, have been related to no progression (28). Our laboratory has used this checkerboard methodology with whole genomic probes in field studies and clinical treatment studies, as well as for the identification of diseased flora in a routine clinical setting (5, 20). This panel includes 12 bacterial species representing both the red and the orange complex. It is clear, however, from recent studies of the human subgingival oral flora based on ribosomal 16S cloning and sequencing that 40% of the bacterial species present are novel species and phylotypes (13, 21). Therefore it seems likely that the presence and significance of a number of these unrecognized periodontal pathogens in the diseased flora remains to be evaluated in comparison with earlier recognized putative pathogens.

The purpose of the present study was to evaluate the association of 13 more recently recognized bacterial species associated with periodontitis ('new panel') in comparison with 12 previously identified putative periodontopathogens ('old panel') using the 'checkerboard' DNA–DNA hybridization method.

Material and methods

Subjects and site selection

Fifty individuals (20 men and 30 women) aged 16–84 years (mean age 54.6 years, SD 11.2 years) referred for treatment of advanced periodontal disease and who fulfilled the inclusion criteria were recruited to the study. Fifty percent of the individuals were smokers.

A clinical examination regarding probing pocket depth and bleeding on probing was performed. Probing pocket depth measurements were recorded parallel to the long axis of the tooth at six location points around the circumference of each tooth as the distance between the gingival margin and the bottom of the probable pocket to the nearest whole millimeter. In conjunction with performing the probing pocket measurement, the area was observed for the presence/absence of bleeding within 30 s (bleeding on probing). The inclusion criteria were as follows:

- at least one site showing probing pocket depth = 6 mm (disease) and bleeding on probing;
- at least one site with probing pocket depth 3 mm without bleeding on probing (health).

The exclusion criteria included antibiotic treatment within the preceding 3 months.

Bacterial sampling

In each subject, one single-rooted tooth was selected which had at least one approximal pocket with probing depth of = 6 mm and bleeding on probing and one tooth with one approximal site with a probing depth of = 3 mm without bleeding on probing. The sample sites were isolated with cotton rolls and supragingival plaque was removed with sterile cotton pellets. One sterile paper point/site (Johnson & Johnson, East Windsor, NJ) was inserted the depth of the periodontal pocket and kept in place for 15 s. The paper points were analyzed by the checkerboard technique (30) modified according to Papapanou et al. (20).

Bacteria and DNA probe development

The samples were transported and processed in the laboratory for the detection of the 12 species constituting the 'old panel' and 13 additional species constituting the 'new panel'. The 25 species used for DNA-probe development are presented in Table 1, with their origin and reference to their association with periodontitis.

The strains were cultured anaerobically on Brucella agar plates for 3–5 days, cells were collected and DNA was extracted with the phenol:chloroform method as described earlier (20). Whole genomic probes were developed with a commercial kit (Boehringer-Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

Checkerboard methodology

The samples were transferred to 100 μl TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.6) and 100 μl 10.5 M NaOH was added and the suspensions boiled for 5 min. After boiling, 800 μl 5 M ammonium acetate was added to each tube and the samples were processed according to standardized procedures (20). The hybrids formed between the bacterial DNA and the probes were detected by application of an antidigoxigenin antibody conjugated with alkaline phosphatase and incubation with a chemiluminiscent substitute (CSPD, Boehringer-Mannheim). Evaluation of the signal was performed at a LumiImager™ workstation (Boehringer-Mannheim) by comparing the obtained signals with those of pooled standard samples containing 10^6 (high-standard) or 10^5 (low standard) of each of the 25 bacterial species. The probes were cross-tested for specificity against the 25 species (Table 1) of the two panels in order to distinguish cross-hybridizations as described by Socransky et al. (29). The obtained chemiluminiscent signals were transformed into a scale of scores from 0 to 5 according to Papapanou et al. (20). The score 1 cut-off was selected to contrast colonized vs. noncolonized sites and the score 3 cut-off to contrast heavily colonized (score 3 or more) vs. noncolonized and less heavily colonized sites.

Statistics

The Chi-squared test was applied for testing the significance of prevalence differences of periodontitis vs. healthy sites.

Results

Using a cutoff level of score 1 (Fig. 1), a 50% prevalence in periodontitis sites was reached for all species of the 'old panel' but only for four species (*Campylobacter gracilis, Prevotella tannerae, Filifactor alocis*, and *Porphyromonas endodontalis*) of the 'new panel' (Table 1). A 90% prevalence was shown in the same sites for *Prevotella intermedia, T. forsythia, Fusobacterium nucleatum, Treponema denticola*, and *Micromonas micros* (previously *Peptostreptococcus micros*) of the 'old panel' and only for *F. alocis* of the 'new panel'.

Only *P. micros* reached 90% prevalence in healthy sites. Generally, species of the 'old panel' showed a higher prevalence than species of the 'new panel' even in the healthy sites. In total, 24 of the 25 tested species (*Dialister pneumosintes* was the exception) showed a higher prevalence in the periodontitis sites than in the healthy sites.

D. pneumosintes was not recorded in any of the samples, although a positive control of the bacteria showed a clear signal, and no cross-hybridizations with the other 24 species were detected.

A significant difference ($P < 0.05$) between periodontitis and healthy sites at this cut-off level was recorded for *P. gingivalis, P. intermedia, Prevotella nigrescens. F. nucleatum, Campylobacter rectus* of the 'old panel', and *Prevotella heparinolytica,*

Table 1. Bacterial strains and origin used for DNA probe development for the 'old' and 'new' panel and checkerboard DNA–DNA hybridization method for test of bacterial markers for periodontitis

Bacterial species	Origin of bacterial strain*	(References) for periodontitis association
Old panel		(13, 21)
Porphyromonas gingivalis	FDC 381	
Prevotella intermedia	ATCC 25611	
Prevotella nigrescens	ATCC 33563	
Tannerella forsythia (Bacteroides forsythus)	ATCC 43037	
Actinobacillus actinomycetemcomitans	FDC Y4	
Fusobacterium nucleatum	ATCC 10953	
Treponema denticola	OMGS 3271	
Micromonas micros (Peptostreptococcus micros)	OMGS 2852	
Campylobacter rectus	ATCC 33238	
Eikenella corrodens	ATCC 23834	
Selenomonas noxia	OMGS 3119	
Streptococcus intermedius	ATCC 27335	
New panel		
Prevotella heparinolytica	CCUG 27827	(2)
Centipeda periodontii	CCUG 44586 (ATCC 35019)	(24)
Selenomonas sputigena	CCUG 44933	(13, 21)
Eubacterium saphenum	ATCC 49989	(13, 21)
Dialister pneumosintes	CCUG 210255	(13, 21)
Bacteroides ureolyticus	CCUG 7319	(13, 21)
Campylobacter gracilis	CCUG 27720	(13, 21)
Prevotella oris	CCUG 15405	(13, 21)
Haemophilus parainfluenzae	CCUG 12836	(13, 21)
Prevotella tannerae	CCUG 34292	(13, 21)
Filifactor alocis	ATCC 35896	(13, 21)
Catonella morbi	CCUG 33640 (ATCC 51271)	(13, 21)
Porphyromonas endodontalis	OMGS 1205	(13, 21)

ATCC, American Type Culture Collection. FDC, Forsyth Dental Center. CCUG, Culture Collection University of Göteborg. OMGS, Oral Microbiology Göteborg Sweden.

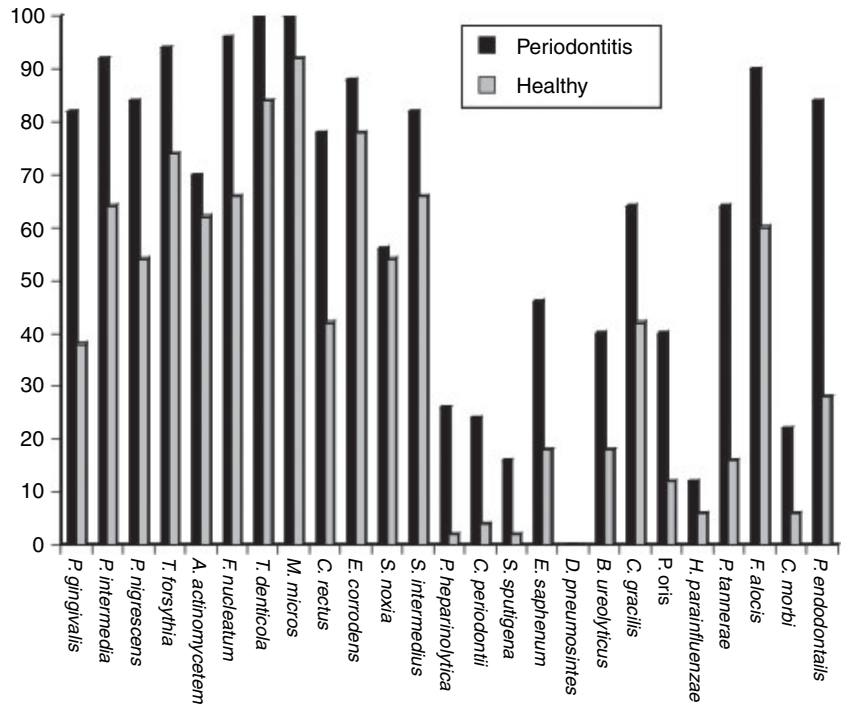

Fig. 1. Prevalence (≥ score 1) of 25 bacterial species from the 'old' and 'new' checkerboard panel.

Centipeda periodontii, Eubacterium saphenum, Prevotella oris. P. tannerae and *P. endodontalis* of the 'new panel'.

Using a higher cut-off level (= score 3) the prevalence was generally lower and exceeded 50% only for *P. intermedia, T. forsythia, T. denticola*, and *M. micros* (Fig. 2). All but two of the 'old panel' species (*Selenomonas noxia*, and *Streptococcus intermedius*) and only *F. alocis* of the 'new panel' reached 30% prevalence at this level. In the healthy sites the prevalence was significantly lower for all species of the 'old panel' and in a few sites, only *P. tannerae* and *F. alocis* of the 'new panel' were detected at this level (score 3). A significant difference ($P < 0.05$) between periodontitis and healthy sites was recorded for none of the 12 'old panel' species. Only *Eikenella corrodens, S. noxia*, and *S. intermedia* did not reach a significant difference. In the 'new panel', only *P. tannerae, F. alocis*, and *P. endodontalis* showed a significance between periodontitis sites and healthy.

Discussion

The main finding in the present study was that most species of the 'old panel' showed a higher prevalence in periodontitis sites (at cut-off 1 or 3) than in healthy (Fig. 1 and 2). The 'new panel' showed the same pattern for several species, although it was less expressed. The difference between periodontitis and healthy sites was greater at a higher bacterial level (cut-off 3).

The 'new panel' was constructed based mainly on the findings of Paster et al. (21) and Kumar et al. (13). In these two studies, samples from various periodontal and gingival pockets were analyzed for specific DNA. The DNA was cloned and sequenced and identified through the gene bank. Many sequences were not identified to phylotypes, but those that were, constituted the basis for the panel in this study. Thus, *E. saphenum, F. alocis,* and *P. endodontalis* were all found by Paster et al. (21) and Kumar et al. (13) at a significantly higher prevalence in periodontitis sites than healthy sites. *P. oris, Selenomonas sputigena*, *Dialister* spp. (strain GBA27), and *C. gracilis* were also found at a higher prevalence in periodontitis cases. *Haemophilus parainfluenzae, P. tannerae*, and *Catonella morbi* were also found to be quite common in periodontitis cases (21). *Bacteroides ureolyticus, P. heparinolytica*, and *C. periodontii* have been found in periodontitis or orofacial infections (2, 6, 24). Most species of the new panel were found in the present

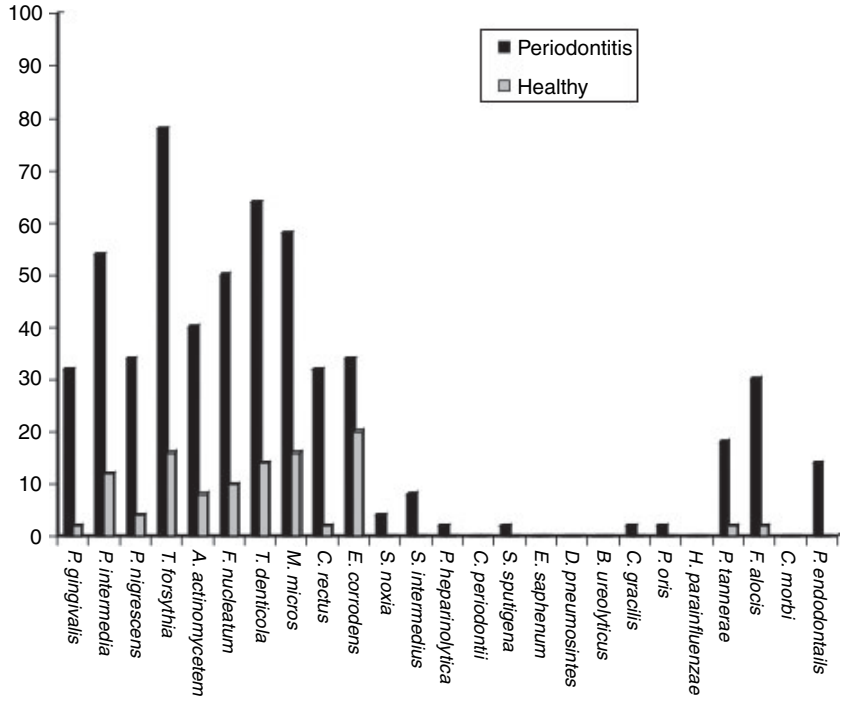

Fig. 2. Prevalence (≥ score 3) of 25 bacterial species from of the 'old' and 'new' checkerboard panel.

study to be more prevalent in sites with deep periodontal pockets compared to shallow pockets (healthy) using the lower cut-off point corresponding to a presence of 10^4 or more, thus in general confirming the findings of Paster et al. (21) and Kumar et al. (13).

D. pneumosintes, however, was not detected in any of the pocket samples in the present study, which is surprising in view of the findings by others (3, 8, 10). The control made by mixing cells of *D. pneumosintes* into negative plaque samples disclosed a positive signal corresponding to the amount of calculated added specific DNA in the sample. It is possible that *D. pneumosintes* was frequently present, however, at low or undetectable levels.

F alocis, P. tannerae, and *P. endodontalis* were more frequently detected in samples from periodontal pockets not only at a lower level (score 1), but also at the higher level (score 3) than in samples from healthy pockets. All three species have been earlier recognized in the human subgingival flora (9, 12, 33, 36), although their association with disease is less well established. *F. alocis* was previously designated as a fusiform-like species but is now recognized as being more related to *Eubacterium* (9). It is described as a gram-positive nonspore forming anaerobic rod, which is slow growing, fastidious, and generally nonreactive in biochemical tests. *F. alocis* has also been recognized as a frequent component in endodontic infections, and was associated with teeth with symptoms (26).

P. tannerae was isolated and described by Moore et al. (17) as a nonmotile, gram-negative rod that fermented carbohydrates and produced succinic and acetic acid. It produces colonies with a tan to black pigment on blood agar. It has also been suggested to be commonly present in endodontic infection according to PCR identification of 16S rRNA genes and has been suggested as a potential pathogen (40). In the present study it did not cross-react in the checkerboard assay with other *Prevotella* spp. including the black-pigmented *P. intermedia* and *P. nigrescens*. Specific virulence factors have not yet been disclosed in this species.

P. endodontalis was first recognized in endodontic infections (31, 36) and taxonomically recognized as its own species already 20 years ago (35). Interestingly, *P. endodontalis* has shown to have an important role in mixed experimental infections (32). Although recognized as an endodontic pathogen, it has also been frequently detected in deep periodontal pockets. This is interesting due to its similarities with the close related *P. gingivalis*, one of the most recognized periodontal pathogens. *P. endodontalis* is taxonomically distinct from *P. gingivalis* based on phenotypic characteristics. *P. endodontalis* is also genotypically quite distinct from *P. gingivalis* and no cross-reaction between the two was noticed using whole genomic probes in the checkerboard method. In conclusion, *P. tannerae, F. alocis*, and *P. endodontalis* were shown to be significantly associated with periodontitis and could serve as markers for a diseased flora and should specifically be tested as markers in periodontitis risk assessments for periodontal progression.

The other bacterial species included in the new panel were not frequently present in the periodontal pocket at a high level (score 3). At a lower level (score 1) they occurred more frequently, but not significantly more frequently than in healthy sites. All the species have been associated previously with periodontal disease and other oral infections. In addition to the association made by Paster et al. (21) and Kumar et al. (13), several other reports have been published. *C. gracilis, C. morbi, C. periodontii, S. sputigena*, and *E. saphenum* have been found in relation to periodontitis (15, 18, 24, 25, 33, 39). *B. ureolyticus* is a common component in orofacial abscesses of odontogenic origin (6). This study could not clearly associate them with the diseased flora in general. It is still possible, however, that they might be of importance in single cases or sites when they occur.

Other species not tested in the present study might also serve as markers for a diseased associated flora. In fact, based on their model of sequencing cloned 16S rDNA inserts, Paster et al. (21) estimated that the bacterial diversity in the subgingival habitat involves 415 species. They also concluded that the encountered taxa were not uniformly distributed in subgingival plaque samples and that some taxa were detected with far greater frequency and in greater numbers than others. Twenty-six of the 306 taxa detected accounted for > 50% of the sequenced clones, while 103 taxa were detected in only one of the 31 subjects (21). It is clearly demonstrated in the present study that all species are not equally prevalent and most of the 'new' species were low, particularly at the higher threshold and only *P. tannerae, F. alocis*, and *P. endodontalis* should be included in the 'old' panel.

Panels as the ones used in the present study may thus function as an optimal panels for identifying a diseased flora. They are likely to detect bacterial species both in diseased and healthy pockets in one and the same patient if very sensitive methods are used and the quantity is not

taken into the consideration. It is clear by using a higher threshold (cut-off level) that most panel bacteria used in the present study are more associated with the diseased periodontal pockets. Consequently, a higher threshold will lower the chance of disclosing the markers in nondiseased pockets (false positives). Together with a high specificity, a method for microbiological diagnostic purposes in the clinic should also have a reasonable simplicity and accuracy. The DNA–DNA hybridization method using whole genomic probes fulfils this demand together with the capacity to report on up to 40 species in a panel at one time (29).

The conclusion of the present study is that the panel of 12 putative periodontal pathogens used for routine diagnostics of the microflora of subgingival samples confirm their association with diseased periodontal pockets. It could be argued that *P. tannerae, F. alocis*, and *P. endodontalis* should be included in this panel.

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