Associations between six DNA probe-detected periodontal bacteria and alveolar bone loss and other clinical signs of periodontitis

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The purpose of the present study was to assess the associations between the presence and amounts of *Actinobacillus actinomycetemcomitans, Bacteroides gingivalis, B. intermedius, Eikenella corrodens, Wolinella recta,* and *Fusobacterium nucleatum* in the periodontal pocket and the degree of alveolar bone loss and other clinical signs of periodontitis, such as probing pocket depth, attachment level, and presence of bleeding on probing at the same site. The study material comprised 16 subjects with or without approximal sites showing longitudinal alveolar bone loss who were selected from a group of 142 subjects monitored radiographically over the past 4 years. In this group 105 sites were examined, of which 58 showed recent alveolar bone loss ≥ 1 mm. Subgingival plaque was collected with absorbent paper points and hybridized with ^{32}P-labeled DNA probes specific for the above-mentioned bacteria. The amount of each bacterial species was correlated with the degree of bone loss over time and the three clinical measurements by means of Spearman rank correlation. *A. actinomycetemcomitans* showed poor correlations with all three clinical signs of periodontal inflammation, whereas *B. gingivalis* and *W. recta* demonstrated significant positive correlations with the three clinical measurements and with attachment level and pocket depth, respectively. In addition, the amount of *A. actinomycetemcomitans, B. gingivalis* and *W. recta* showed significant positive correlation with the extent of alveolar bone loss at the site. In contrast, the amounts of *B. intermedius, E. corrodens,* and *F. nucleatum* showed negative correlations with all four measurements. *B. gingivalis* and *W. recta* demonstrated stronger associations when their amounts in the pocket were combined and when proportions relative to the amount of *B. intermedius, E. corrodens,* and *F. nucleatum* at the site were assessed. Thus, the results support the concept that specific combinations and relations of organisms rather than single organisms may play an important role in the pathogenesis of periodontitis. □ *A. actinomycetemcomitans; bacteria; B. gingivalis; B. intermedius. DNA probes; E. corrodens; F. nucleatum; nucleic acid probes; periodontal disease; radiographs; W. recta*

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There has long been a controversy concerning the specific role of certain bacteria in the pathogenesis of periodontal diseases (1). Several oral microorganisms have been proposed to play an important role in this context (2–5), of which *Actinobacillus actinomycetemcomitans, Bacteroides gingivalis,* and *B. intermedius* are most often mentioned (6). *Wolinella recta, Eikenella corrodens,* and *Fusobacterium nucleatum* are also thought to be related to periodontal diseases (7). However, the presence of *A. actinomycetemcomitans, B. gingivalis,* and *B. intermedius* in a periodontal pocket has been found to be a poor indication for the occurrence of significant attachment loss at that site 1 year later (8).

Bacterial culturing, often used for the detection of periodontal pathogens, is a laborious process and has several shortcomings (9). Nucleic acid probes have recently been introduced as new tools in the diagnostic microbial laboratory, and their application in periodontology has been discussed (10). These probes are often more efficient than cultural methods (11) and more reliable than currently available biochemical tests (12, 13).

The purpose of the present investigation was to use DNA probes specific for *A. actinomycetemcomitans, B. gingivalis, B. intermedius, E. corrodens, W. recta,* and *F. nucleatum* to study the association between the amounts of these six microorganisms resident in subgingival sites and the extent of the alveolar bone loss that had taken place over the past 4 years and various clinical indicators of periodontitis, such as probing pocket depth, level of attachment, and presence of bleeding on probing at the same sites.

Material and methods

Study population

A group of 142 subjects, who had been included in a previous study of periodontal disease progression (14), was monitored radiographically over a period of 4 years, using a standardized periapical technique (15). From this group 13 subjects who had had one or more periodontal sites with significant alveolar bone loss during the past 4 years were selected for the present study. In addition, three subjects who had no interproximal sites with alveolar bone loss during the same interval were included. Nine of the subjects were men and seven were women, and their ages ranged from 33 to 71 years (mean, 57 years). None of these subjects were receiving systematic periodontal treatment or were given antibiotics during the monitoring period.

Periapical radiographs taken at base line and 4 years later were examined, and the alveolar bone height in the interproximal areas of teeth was assessed in accordance with a previously described method (15, 16). Six dry skulls were used to investigate the reproducibility and the validity of the radiographic technique used (17). The sum of the error variances due to the measurement technique and the repeated radiographic exposures taken 1 week apart was 0.1. On the basis of a previously suggested criterion (18, 19), significant alveolar bone loss was defined as ≥1 mm height reduction in the alveolar bone crest assessed on the two consecutive radiographs.

All sites showing alveolar bone loss during the observation period were examined clinically and bacteriologically. In addition, sites without bone loss during the same period and with various pocket depths, attachment losses, and with or without bleeding on probing in each subject were assessed.

Clinical examination

For each site, three clinical variables were assessed at the end of the 4 years and used as indicators of periodontal disease. These included probing pocket depth, probing attachment level, and presence of bleeding on probing. Measurement of the attachment level and the pocket depth followed the guidelines given by Glavind & Löe (20). The measurements were made by one examiner using approximately 50 ponds of probing force and calibrated periodontal probes. Values were rounded off to the nearest full millimeter.

Subgingival plaque sampling

The sites to be sampled were isolated with cotton rolls, and sterile pledgets of cotton were used to remove the supragingival plaque. A sterile medium-sized absorbent paper point (Johnson & Johnson) was then inserted into the depth of the periodontal pocket/sulcus and pushed gently toward the apical extension of the pocket/sulcus, kept there for 10 sec, and then removed and placed in a sterile dry Eppendorf tube, which was sealed with paraffin paper.

Sample preparation

The bacteria of the plaque were identified by the method described by French et al. (21). Each bacterial sample was eluted from the paper point, and the bacterial DNA was then denatured and equilibrated with an equal volume of loading buffer (1:1 3M NaCl, 0.3M NaOH–2M NH_4OAc). Aliquots of the prepared samples were then immobilized by applying them to a nitrocellulose filter placed in a Minifold II Slot Plot apparatus (Schleicher & Schuell). Vacuum was applied to bring the denatured DNA onto the filters. The nitrocellulose filters were then rinsed in 0.5M NaCl, air-dried, and baked at 80°C for 1 h. The filters were prehybridized in 5X SSC (SSC = 0.15 M NaCl, 0.015 M citrate, pH 7.0), 0.5% sodium dodecyl sulfate (SDS), 100 μg/ml salmon sperm DNA, and 5X Denharts, for 1 h at 65°C.

Bacterial detection using DNA probes

The present study utilized ^{32}P-labeled DNA probes developed at BioTechnica Diagnostics, Cambridge, Mass., USA, as previously described (21–23). These probes comprised a cloned *A. actinomycetemcomitans*-specific probe and whole genomic DNA probes against *B. intermedius, B. gingivalis, E. corrodens, W. recta* and *F. nucleatum*. It has been shown that these probes can detect 10^2–10^3 cells of bacteria in a plaque sample (21) and possess high specificity, as indicated when tested for cross-reactivity to a battery of oral microorganisms (21, 23; J. A. Lippke, W. J. Peros, M. W. Kelville, et al. Unpublished observations).

Samples immobilized on the nitrocellulose filters were hybridized with the probes in accordance with French et al. (21). The filters were hybridized for 3.5 h at 65°C, washed extensively, air-dried, and exposed to Kodak X-omat AR film with an intensifying screen at −70°C for 48 h. The film was then developed in an automatic film developer.

Reference controls

As a reference for assessing the bacterial cell counts in each test sample, predetermined quantities of strains of *A. actinomycetemcomitans, B. intermedius, B. gingivalis, E. corrodens, W. recta* and *F. nucleatum* were prepared (24) and used as positive controls. The organisms were grown from stock cultures that had been biochemically confirmed and lyophilized in aliquots for future use. For each of the six strains to be identified, three samples were prepared from these lyophilized stocks, each containing 10^3, 10^4, or 10^5 cells per slot aliquot as determined by optical density measurements. In addition, a negative control specimen consisting of a well-characterized strain of *Actinomyces viscosus* (10^5 cells) was prepared for each of the six species in the same manner. Triplicates of the positive and the negative control specimens were then treated for immobilization and hybridized with DNA probes as described above.

DNA probe assay scoring

The signals of the test samples on the X-ray film were compared with those from the predetermined quantities of sample strains of *A. actinomycetemcomitans, B. intermedius, B. gingivalis, E. corrodens, W. recta,* and *F. nucleatum* on the same film. In this manner the intensities of the signals from the test samples could be compared to the intensity of the signal from each of the three positive control specimens. Signals were assigned one of the following scores: 0 = less than 10^3; 1 = more than or equal to 10^3 but less than 10^4; 2 = more than or equal to 10^4 but less than 10^5; 3 = more than or equal to 10^5.

Repeated scoring of the test assays was done within 1 week, to assess the reliability of the readings. The scoring was identical in 97.8% of the readings. The kappa and the Spearman coefficients between the repeated measurements were 0.967 and 0.983, respectively.

Data analysis

The relationship between the amount of each of the six organisms in the pocket/sulcus and the alveolar bone height reduction during the past 4 years and the clinical measurements of pocket depth, attachment level and presence of bleeding on probing at the same site was assessed by means of Spearman's rank correlation. Both the number of organisms and proportion of these numbers to the total number of all six organisms at the site were assessed and used to indicate the amounts and the relative amounts to totals, respectively. The resulting correlation matrix was studied, and organisms showing negative correlation coefficients were recognized. The amounts of organisms indicating positive correlation

Table 1. Correlations between amounts of *A. actinomycetemcomitans* (*Aa*), *B. gingivalis* (*Bg*), *W. recta* (*Wr*), *B. intermedius* (*Bi*), *E. corrodens* (*Ec*), and *F. nucleatum* (*Fn*) and three clinical indicators of periodontitis and alveolar bone loss over 4 years, measured at the same sites

Type of bacteria	Attachment level		Pocket depths		Bleeding on probing		Alveolar bone loss	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Aa	0.12	NS	0.18	NS	0.05	NS	0.51	**
Bg	0.30	**	0.32	**	0.24	*	0.23	*
Wr	0.24	*	0.34	**	0.17	NS	0.27	**
Bi	−0.30	**	−0.25	*	−0.26	*	−0.08	NS
Ec	−0.12	NS	−0.21	*	−0.09	NS	−0-09	NS
Fn	−0.08	NS	−0.09	NS	−0.05	NS	−0.13	NS

r = Spearman's correlation coefficient. NS = not significant ($P > 0.05$); * = $P < 0.05$; ** = $P < 0.01$.

coefficients were then computed relative to the amount of organisms with negative correlations and correlated with the clinical and the radiographic measurements.

The *t* values were computed by the following formula:

$$t = \frac{(n-2)^{1/2}\, r}{(1-r^2)^{1/2}},$$

where r = Spearman correlation coefficient.

The probability values were obtained by treating *t* as coming from a *t* distribution with $n - 2$ degrees of freedom.

Results

A total of 105 sites in the 16 subjects were examined. Of these sites 58 (55%) showed significant alveolar bone loss over the past 4 years. There were significant positive correlations between the amount of *B. gingivalis* and all three clinical measurements and between the amount of *W. recta* and the attachment level and the probing pocket depth (Table 1). In addition, the amounts of *A. actinomycetemcomitans, B. gingivalis,* and *W. recta* in the pocket/sulcus showed significant positive correlation with the degree of alveolar bone loss which had taken place at the same site over the past 4 years. In contrast, the amounts of *B. intermedius, E. corrodens,* and *F. nucleatum* showed negative coefficients when correlated with all four measurements (Table 1).

However, most correlations were potentiated when the amount of each organism was assessed relative to the total amount of all six organisms at the site (Table 2),

Table 2. Correlations between the relative amounts of *A. actinomycetemcomitans* (*Aa*), *B. gingivalis* (*Bg*), *W. recta* (*Wr*), *B. intermedius* (*Bi*), *E. corrodens* (*Ec*), and *F. nucleatum* (*Fn*) to the total amount of all six bacterial species, and three clinical indicators of periodontitis and alveolar bone loss over 4 years, measured at the same sites

Type of bacteria	Attachment level		Pocket depths		Bleeding on probing		Alveolar bone loss	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Aa	0.06	NS	0.10	NS	0.08	NS	0.20	NS
Bg	0.40	**	0.43	**	0.32	**	0.34	**
Wr	0.34	**	0.42	**	0.06	NS	0.34	**
Bi	−0.52	**	−0.47	*	−0.24	*	−0.24	*
Ec	−0.26	*	−0.40	**	−0.23	*	−0.33	**
Fn	−0.22	*	−0.27	**	−0.10	NS	−0.35	**

r = Spearman's correlation coefficient. NS = not significant ($P > 0.05$); * = $P < 0.05$; ** = $P < 0.01$.

Table 3. Correlations between the relative amounts of *A. actinomycetemcomitans* (*Ac*), *B. gingivalis* (*Bg*), and *W. recta* (*Wr*) to the total amount of *B. intermedius, E. corrodens*, and *F. nucleatum* at the site, and three clinical indicators of periodontitis and alveolar bone loss over 4 years, measured at the same sites

Type of bacteria	Attachment level		Pocket depths		Bleeding on probing		Alveolar bone loss	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Aa	0.07	NS	0.03	NS	0.06	NS	0.43	**
Bg	0.51	**	0.48	**	0.36	**	0.36	**
Wr	0.43	**	0.50	**	0.21	*	0.39	**

r = Spearman's correlation coefficient. NS = not significant ($P > 0.05$); * = $P < 0.05$; ** = $P < 0.01$.

and still stronger correlations resulted for *B. gingivalis* and *W. recta* when their amounts were assessed relative to the total amount of *B. intermedius, E. corrodens*, and *F. nucleatum* in the pocket/sulcus (Table 3). This was true for all organisms but *A. actinomycetemcomitans*, whose correlation coefficient faded when its amount was assessed relative to the amount of *B. intermedius, E. corrodens* and *F. nucleatum* (Tables 2 and 3).

Similar patterns were seen when the amounts of two or more organisms at the site were combined and correlated with the measured variables. Of the organisms with positive correlations, *B. gingivalis* and *W. recta* showed stronger correlations than did the other combinations (Table 4), whereas combining *B. intermedius, E. corrodens*, and *F. nucleatum* seemed to increase the strength of the negative correlations (Table 5).

Discussion

The study population was originally selected for a longitudinal radiographic study of the development of periodontal bone loss in subjects with various degrees of periodontal health and disease. For the purpose of the present study this may constitute extra difficulties in the interpretation of the data. However, this approach may be more relevant to the situation among people who cannot readily be regarded as periodontal patients.

The relationship between the amounts of six DNA probe-detected periodontal microorganisms and three clinical variables used as indicators of periodontitis and the reduction in the crestal alveolar bone height in proximal sites over the past 4 years was studied in the present material. The results indicated that the amounts of *B. gingivalis* and of *W. recta* in the periodontal pockets were significantly associated with the clinical signs of periodontitis and with the amount of alveolar bone loss at the sites, whereas *A. actinomycetemcomitans* showed a significant association with the bone loss only, and this association was stronger than those demonstrated by *B. gingivalis* and *W. recta* separately. Further, the findings indicated that the associations displayed by *B. gingivalis* and *W. recta* were potentiated when their amounts were calculated as proportions of all six organisms in the pocket and were further strengthened when proportions to the total amount of *B. intermedius, E. corrodens*, and *F. nucleatum* at the site were assessed.

Negative correlation coefficients were demonstrated in the present study when the amounts of each of *B. intermedius, E. corrodens*, and *F. nucleatum* in the pocket were correlated with probing pocket depth, attachment level, bleeding on probing, and the amount of alveolar bone loss at the site. Tanner et al. (25) also reported negative correlations between the amount of *E. corrodens* and the periodontal inflammation. It is noteworthy that the results of the present study were meaningful in the sense that the organisms yielded analogous and consistent results when correlated with the four variables, and organisms showing positive or

Table 4. Correlations between combinations of *A. actinomycetemcomitans* (*Aa*), *B. gingivalis* (*Bg*), and *W. recta* (*Wr*) at the site and three clinical indicators of periodontitis and the alveolar bone loss over 4 years, measured at the same sites. For each site, amounts of the bacterial combinations (no.), the ratio of these amounts to the total amount of all six species (%total), and the ratio of the amounts to the total amount of *B. intermedius*, *E. corrodens*, and *F. nucleatum* (%*Bi, Ec, Fn*) were used

Type of bacterial combination	Attachment level						Pocket depths						Bleeding on probing						Alveolar bone loss					
	No.		% total		%*Bi, Ec, Fn*		No.		% total		%*Bi, Ec, Fn*		No.		% total		%*Bi, Ec, Fn*		No.		% total		%*Bi, Ec, Fn*	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Aa + Bg	0.35	**	0.48	**	0.55	**	0.33	**	0.46	**	0.50	**	0.22	*	0.25	*	0.29	**	0.26	**	0.35	**	0.39	**
Aa + Wr	0.22	*	0.32	**	0.45	**	0.31	**	0.42	**	0.49	**	0.11	NS	0.14	NS	0.22	*	0.24	*	0.32	**	0.37	**
Bg + Wr	0.28	**	0.50	**	0.53	**	0.35	**	0.48	**	0.55	**	0.23	*	0.31	**	0.30	**	0.28	**	0.47	**	0.50	**
Aa + Bg + Wr	0.28	**	0.50	**	0.60	**	0.34	**	0.54	**	0.61	**	0.18	NS	0.35	**	0.35	**	0.28	**	0.40	**	0.44	**

r = Spearman's correlation coefficient. NS = not significant ($P > 0.05$); * = $P < 0.05$; ** = $P < 0.01$.

negative correlations with one measurement tended to show similar results with the other measurements.

A. actinomycetemcomitans has often been recovered from, and associated with, sites undergoing active periodontal breakdown (26). Nevertheless, such sites may look clinically healthy (27). The results of the present study are consistent with these reports, since they demonstrated poor correlations between the amount of this organism and the clinical indicators of periodontitis at the site. However, *A. actinomycetemcomitans* was significantly associated with the degree of alveolar bone loss, and even stronger correlation emerged when its amount was not assessed relative to the other organisms tested. These findings may indicate that *A. actinomycetemcomitans* possibly possesses a periodontal pathogenic mechanism distinct from that of the other organisms, which is consistent with recent findings on *A. actinomycetemcomitans*-residing phages (28).

Combinations of organisms inhabiting the same site have been suggested to be associated with some clinical indicators of periodontitis (29, 30). Most of these combinations were based on grouping sites by means of similarity coefficients (31), resulting in poorly defined microbial complexes. The complex nature of the microbial flora residing in the periodontal pocket and the tremendous task involved in attempting to define microbial interactions here should, however, be appreciated. So far, our findings tend to cluster the six organisms probed in the present study into three groups. The first group is formed by *A. actinomycetemcomitans*, which does not seem to be influenced by the other five organisms. When the quantity of this organism was used in combination with that of *B. gingivalis* or *W. recta*, no stronger correlations resulted, which may indicate that the latter two organisms had no additive effect on the pathogenic role of *A. actinomycetemcomitans* in periodontitis. In addition, *A. actinomycetemcomitans* probably plays a significant role irrespective of the amount of *B. intermedius, E. corrodens,* and *F. nucleatum* in the site. The other group, which consisted of *B. gingivalis* and *W. recta*, both correlating positively with the

Table 5. Correlations between combinations of *B. intermedius* (*Bi*), *E. corrodens* (*Ec*), and *F. nucleatum* (*Fn*) at the site and three clinical indicators of periodontitis and the alveolar bone loss over 4 years, measured at the same sites. For each site, amounts of the bacterial combinations (no.) and the proportion of these amounts to the total amounts of all six species (%total) were used

Type of bacterial combination	Attachment level				Pocket depths				Bleeding on probing				Alveolar bone loss			
	No.		% total		No.		% total		No.		% total		No.		% total	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Bi + *Ec*	−0.15	NS	−0.43	**	−0.13	NS	−0.50	**	−0.08	NS	−0.30	**	−0.05	NS	−0.30	**
Bi + *Fn*	−0.24	*	−0.54	**	−0.17	NS	−0.50	**	−0.18	NS	−0.21	*	−0.04	NS	−0.31	**
Ec + *Fn*	−0.02	NS	−0.21	*	0.01	NS	−0.30	**	−0.03	NS	−0.17	NS	−0.08	NS	−0.35	**
Bi + *Ec* + *Fn*	−0.11	NS	−0.47	**	−0.08	NS	−0.53	**	−0.04	NS	−0.23	*	−0.06	NS	−0.47	**

r = Spearman's correlation coefficient. NS = not significant ($P > 0.05$); * = $P < 0.05$; ** = $P < 0.01$.

extent of periodontal inflammation and the bone loss and showing even stronger correlations when their amounts were combined, may have an additive effect on each other and may possibly interact with the third group, which comprised those demonstrating negative correlations.

Possible mechanisms for such interactions have recently been discussed by Socransky et al. (32), who suggested three likely mechanisms, consisting of a 'key' pathogen, 'mixed infections', and additive and synergistic interactions. The tendency for large numbers of *B. intermedius*, *E. corrodens*, and *F. nucleatum* to weaken the associations between *B. gingivalis* and *W. recta* and the periodontal disease activity at a site, together with the additive effect of combinations of the latter two organisms, tend to support the third mechanism.

There have been conflicting results from previous studies on the importance of *B. intermedius* in periodontitis (for a review, see Ref. 33). One possible explanation is that this organism is currently classified into two groups (34), whereas a third group has recently been reported (35). In addition, some results indicate that *B. intermedius* type II may be more important than type I in the pathogenesis of periodontal diseases (36). Similarly, Slots et al. (11) used an immunofluorescence technique against two serotypes of *B. intermedius* and found no significant association between this organism and the attachment level changes, whereas a significant association was demonstrated when *B. gingivalis* was tested. As a result of the broadening of our present knowledge of taxonomy, the group of organisms formerly referred to as black-pigmented *Bacteroides* has now been classified into diverse subspecies. This makes comparison of our results with those of earlier studies on black-pigmented *Bacteroides* difficult.

The retrospective nature of the present study and the fact that the microflora was not examined throughout the study period must be kept in mind during interpretation of the results. Thus, some of the microorganisms detected may have been established after the bone loss had occurred. It is obvious, however, that designing a longitudinal study to monitor the bacterial flora of sites undergoing progressive periodontal destruction is a difficult task, since there is currently no accurate method to predict which sites are going to become or are active (37). It has been found that even the presence of *A. actinomycetemcomitans*, *B. gingivalis*, or *W. recta* at a site is a poor indicator of the prediction of further deterioration at that site during the next year (8). However, our results indicate that the amounts of *B. gingivalis* and *W. recta* in the periodontal pocket are significantly associated with clinical signs of periodontitis, such as pocket depth, attachment level, bleeding on probing, and the bone loss at the site, and that these associations are strengthened when the amounts of these organisms are combined or when considered in relation to the amount of *B. intermedius*, *E. corrodens*, and *F. nucleatum* in the pocket. These findings may indicate that *B. gingivalis* and *W. recta* probably are more strongly associated with the periodontal disease activity when *B. intermedius*, *E. corrodens*, and *F. nucleatum* are absent or present in small amounts. It may also be postulated that detecting large proportions of *B. intermedius*, *E. corrodens*, and *F. nucleatum* in the periodontal pocket may reflect absence or a low degree of periodontal inflammation and recent alveolar bone loss at the site. These findings support the concept that certain combinations of organisms may be important to the development of periodontitis rather than the individual action of a specific agent. Further, *A. actinomycetemcomitans* seems to be strongly associated with recent disease activity but not with present signs of periodontal inflammation.

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