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Disease Severity Associated with Presence in Subgingival Plaque of *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Tannerella forsythia*, Singly or in Combination, as Detected by Nested Multiplex PCR[▽]

D. Ready,[1]* F. D'Aiuto,[2] D. A. Spratt,[2] J. Suvan,[2] M. S. Tonetti,[3] and M. Wilson[2]

Eastman Dental Hospital, UCLH NHS Foundation Trust,[1] and UCL Eastman Dental Institute, University College London,[2] 256 Gray's Inn Road, London WC1X 8LD, United Kingdom, and European Research Group on Periodontology, Bern, Switzerland[3]

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This study used a nested multiplex PCR method to detect three periodontal pathogens in subgingival plaque collected before treatment and at 2 and 6 months posttreatment from 107 patients with severe, generalized periodontitis. The proportions of the patients who harbored these bacteria before periodontal treatment were as follows: *Tannerella forsythia*, 81%; *Porphyromonas gingivalis*, 78%; and *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans*, 47%. At 2 months posttreatment there was a significant reduction in the numbers of patients harboring *P. gingivalis* (46%; $P < 0.001$) or *T. forsythia* (63%; $P = 0.043$) but not *A. actinomycetemcomitans* (50%) compared to pretreatment data. At 6 months posttreatment, significantly fewer patients harbored *P. gingivalis* (43%; $P < 0.001$); *A. actinomycetemcomitans*, (31%; $P = 0.025$), or *T. forsythia* (63%; $P = 0.030$). Interestingly, at baseline and at 2 months posttherapy, subjects who harbored only a single pathogen had a greater level of periodontal disease than subjects who harbored two, or all three, of these periodontal pathogens. These data suggest that a reduction in the number of species present may be associated with an increase in the severity of periodontal diseases.

Periodontal diseases are among the most widespread bacterial diseases of mankind and are estimated to affect 10 to 15% of the population (7). Periodontitis is an inflammatory disease of the supporting structures of the teeth, which can lead to destruction of the associated tissues. Approximately 630 different taxa (12) are present in the oral cavity; however, relatively few are considered important in the etiology of periodontitis. Indeed, clinical studies have identified only 10 to 15 bacterial species which are regarded as potential periodontal pathogens in adults (10, 23). Three of these bacteria, *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans*, *Tannerella forsythia* (formerly *Bacteroides forsythus*), and *Porphyromonas gingivalis*, were officially designated etiological agents of periodontitis in 1996 (2). The presence of these bacteria has been shown to be a useful indicator of active disease and of increased risk of gingival attachment loss. A wide range of techniques have been used to detect these periodontal pathogens, including bacterial culture, immunofluorescence, enzyme-linked immunosorbent assay, enzyme assays, and DNA probes; however, the culture techniques are time-consuming and often require a 7-day period before any results are available, whereas the nonculture techniques may suffer from a lack of sensitivity or specificity. The introduction of molecular techniques including the PCR, particularly nested PCR, has lowered the routine threshold of bacterial detection to as few as 10 cells (9, 22). Most PCR methods detect only a single species at a time; however, by the combination of multiple primers in a single reaction, it is possible to detect more than one bacterial species in a single patient sample. A PCR technique involving a set of primers that targets species-specific regions of the 16S rRNA genes of *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythia* has been developed to demonstrate the presence of these periodontal pathogens (22), and by using a nested methodology, as adapted by Gafan et al. (9), the detection limit has been reduced. Although the use of a multiplex PCR method for the detection of these periodontal pathogens has been reported (9, 22), those studies have concentrated on healthy subjects, children with gingivitis, and a small number of patients with early adult periodontits (9, 22). The aims of the present study were (i) to use a nested multiplex PCR method to determine any effect of periodontal therapy on the presence of *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythia* in 107 adults with severe generalized periodontitis; (ii) to determine any associations between clinical parameters and the presence of these periodontal pathogens; and (iii) to ascertain the predictive value of detection of these periopathogens on response to periodontal therapy or further periodontal disease progression over a 6-month period.

MATERIALS AND METHODS

Subjects. Samples were derived from a clinical study performed at the Periodontology Department of the University College London Hospitals NHS Foundation Trust (8). Briefly, this was a prospective, longitudinal trial with 6-month follow-up. Participants were recruited from subjects referred to the unit. Subjects presenting with severe, generalized periodontitis were invited to participate in the study (3). Exclusion criteria included (i) known systemic diseases, (ii) history and/or presence of other infections, (iii) systemic antibiotic treatment in the

* Corresponding author. Mailing address: Microbial Diseases, Eastman Dental Hospital, UCLH NHS Foundation Trust, 256 Gray's Inn Road, London WC1X 8LD, United Kingdom. Phone: 44 (0) 20 7915 1050. Fax: 44 (0) 20 7915 1127. E-mail: d.ready@eastman.ucl.ac.uk.
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TABLE 1. Mean clinical data at baseline and 2 and 6 months after completion of the nonsurgical periodontal therapy

Sampling time	Full-mouth subgingival plaque score		Full-mouth bleeding score		Full-mouth PPD (mm)		No. of pockets ≥5 mm	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Baseline	58.0	1.9	63.6	1.5	4.4	0.06	80.2	2.3
2 mo posttherapy	20.9[a]	1.5	15.8[a]	1.2	3.3[a]	0.05	27.8[a]	1.7
6 mo posttherapy	20.1[a]	1.1	17.1[a]	1.2	3.2[a]	0.05	22.9[a]	1.5

[a] Significant reduction in the clinical measurement compared to baseline data ($P < 0.01$).

preceding 3 months, and (iv) pregnancy or lactation in females. All patients gave written informed consent, and the study had been reviewed and approved by the Eastman/University College London Hospitals' joint ethics committee. A baseline visit was conducted by a blind calibrated examiner to collect a complete medical history and standard clinical periodontal parameters (plaque and bleeding scores, periodontal probing depths [PPD], number of periodontal pockets of ≥5 mm, recession of the gingival margin, and clinical attachment levels [CAL]). Patients underwent a standard phase of nonsurgical periodontal treatment that was performed by a periodontologist and were reexamined at 2 and 6 months after completion of this treatment.

Sample collection and DNA isolation. A pooled sample of subgingival periodontal plaque was collected from the four deepest periodontal pockets (one in each quadrant of the mouth) before periodontal therapy commenced. Samples at 2 and 6 months posttherapy were collected from the same sample sites; in the absence of a periodontal pocket, samples were collected from the appropriate area of the gingival sulcus. The subgingival plaque was collected using a sterile curette and immediately placed in a sterile container with 1 ml of reduced transport fluid (20). The plaque samples were then dispersed in the reduced transport fluid by vortexing for 60 s. The whole genomic DNA was extracted from 500 μl of this sample using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN), and the genomic DNA was then stored at −20°C for further analysis.

16S rRNA gene PCR (first round). The whole genomic DNA extracts were used as templates in a PCR technique using the universal primers that targeted the 16S rRNA gene. The forward primer (27F) had a nucleotide sequence of 5′-AGAGTTTGATCMTGGCTCAG-3′ and the reverse primer (1492R) of 5′-TACGGYTACCTTGTTACGACTT-3′ (Genosys, Cambridgeshire, United Kingdom) (14), with an expected amplicon size of 1,465 bp. The reaction consisted of 30 amplification cycles of: 94°C for 1 min, 54°C for 1 min, and 72°C for 1.5 min. Initial dissociation of DNA was for 5 min at 95°C, and the final primer extension was for 5 min at 72°C. Each PCR was carried out with a negative control consisting of sterile deionized water in addition to a positive control consisting of DNA extracted from a pure culture of *Escherichia coli* (NCTC 10418; 10 ng/μl). The amplification products were visualized on a 1% agarose gel (Amresco, Solon, OH) and compared with a 100-bp DNA molecular size marker (Promega).

Multiplex PCR (second round). The 16S rRNA gene PCR products were then used as templates for the multiplex PCR using one universal reverse primer and three species-specific forward primers, with a detection limit of 10 cells/ml for any of the three pathogens, as described previously (9). The primers chosen for the detection of the three putative pathogens targeted specific regions within the 16S rRNA gene. The expected product lengths were 197 bp for *P. gingivalis*, 360 bp for *A. actinomycetemcomitans*, and 745 bp for *T. forsythia*. The nucleotide sequences for the four selected primers were as follows: *P. gingivalis* specific forward primer (PgF), 5′-TGTAGATGACTGATGGTGAAAACC-3′; *A. actinomycetemcomitans* specific forward primer (AaF), 5′-ATTGGGGTTTAGCCC TGGTG-3′; *T. forsythia*-specific forward primer (TfF), 5′-TACAGGGGAATA AAATGAGATACG-3′; and the conserved reverse primer (ConR), 5′-ACGTC ATCCCCACCTTCCTC-3′ (Genosys) (9, 22). The final volume of each PCR mixture was 53.6 μl (comprising 48.6 μl of the master mixture and 5 μl of DNA template). A hot-start step was included in this protocol, and AmpliTaq Gold (Applied Biosystems, Foster City, CA) was used. The master mixture comprised 10.3 mM Tris-HCl, 51.3 mM KCl (10× PCR buffer II), 2.9 mM $MgCl_2$, 0.15 μM primer AaF, 0.74 μM primer TfF, 0.49 μM primer Pgf, 0.47 μM primer ConR, and 10U of AmpliTaq gold. The deoxynucleoside triphosphates included dATP, dCTP, and dGTP, each at 0.2 mM, and 600 mM dUTP (Promega, Southampton, United Kingdom). The cycling parameters consisted of 40 cycles of 95°C for 1 min, 61°C for 1 min, and 72°C for 5 min. Initial dissociation of DNA was for 10 min at 95°C, and the final primer extension was for 10 min at 72°C. Each PCR was carried out with a negative control consisting of sterile deionized water as well as a positive control consisting of genomic DNA extracted from pure cultures of *P. gingivalis* NCTC 11834, *A. actinomycetemcomitans* NCTC 9710, and *T. forsythia* ATCC 43037, all at a final concentration of 10 ng/μl. Post-PCR analysis was carried out by electrophoresis of the PCR products on a 2.3% agarose gel. For quality control purposes, 10% of the samples were retested to confirm that consistent results were obtained. Additionally, 5% of the PCR products were randomly selected and excised from agarose gels, and the DNA was purified using a QIAquick gel extraction kit (Qiagen Ltd., Crawley, United Kingdom); these products were then sequenced using BigDye terminator cycle (AB Biosystems, Foster City, CA) and analyzed using a 310 Genetic Analyzer (AB Biosystems, Foster City, CA). The DNA sequences were analyzed online to confirm species identification (NCBI Blast; http://www.ncbi.nlm.nih.gov /BLAST).

Statistical methods. Data were expressed as mean ± standard error unless differently specified. Clinical periodontal parameters, as well as frequency of detection of pathogens, were all normally distributed, and therefore parametric testing was used. The null hypothesis of these data analyses was that no difference in the frequency of detection of periodontal pathogens would be observed following 2 and 6 months of periodontal therapy. The McNemar paired test was used. Secondary outcomes included possible associations between detection of one or multiple periodontal pathogens and other clinical periodontal parameters at each study visit (baseline and 2 and 6 months) and predictive value of the presence of periodontal pathogens on subsequent disease progression following therapy. Indeed, we created multivariate analysis-of-variance models to ascertain the differences in clinical continuous periodontal parameters (probing pocket depth, number of periodontal pockets, and whole-mouth gingival and plaque scores) among subgroups of patients with positive detection of one or more periodontal pathogens. Differences in age, gender, ethnicity, and cigarette smoking were accounted for in each variance model. Between-group comparisons were performed with Bonferroni corrections to account for multiple testing. The level of statistical significance was set at a P value of <0.05. The statistical package used was SPSS version 14.0 (SPSS Inc., Chicago, IL).

RESULTS

Nonsurgical periodontal therapy resulted in a significant improvement of all clinical periodontal parameters at 2 and 6 months posttherapy (Table 1). A mean reduction in the number of periodontal pockets that were ≥5 mm ($P < 0.01$) was obtained, together with a reduction in the mean full-mouth PPD and full-mouth bleeding ($P < 0.01$) and plaque ($P < 0.01$) scores at 2 and 6 months post therapy compared to baseline data.

The presence of a clear band of the expected size was recorded as a positive result. After the first round of PCR, the 16S rRNA gene was detected in 107 of the 109 baseline samples and in 93 and 95 of the 96 samples collected at 2 and 6 months posttherapy, respectively. The six samples in which no 16S rRNA gene products could be detected were excluded from this study. All of the sequence data from the excised amplicons showed complete agreement with the assumed species identification. All of the samples which were randomly reanalyzed gave identical results for the presence or absence of these

TABLE 2. Numbers of subjects harboring *A. actinomycetemcomitans, P. gingivalis*, or *T. forsythia*

Pathogen	No. (%) of subjects positive[a] at:		
	Baseline (n = 107)	2 mo (n = 93)	6 mo (n = 95)
A. actinomycetemcomitans	50 (46.7)	46 (49.5)	29 (30.5)*
P. gingivalis	83 (77.6)	43 (46.2)**	41 (43.2)**
T. forsythia	87 (81.3)	59 (63.4)*	60 (63.2)*

[a] Asterisks indicate a significant reduction in the number of subjects harboring a periodontal pathogen compared to baseline data. *, $P < 0.05$; **, $P < 0.01$.

periopathogens each time the sample was tested. DNA products were not observed in any of the negative control samples.

Table 2 shows the prevalences of *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythia* in patients with generalized aggressive periodontitis before treatment and at 2 and 6 months posttreatment. The most prevalent of the three bacteria detected in subgingival plaque from these patients was *T. forsythia*, followed by *P. gingivalis* and *A. actinomycetemcomitans*. Throughout the 6-month period, *T. forsythia* was detected in 81.3% to 63.2%, *P. gingivalis* in 77.6% to 43.2%, and *A. actinomycetemcomitans* in 49.5% to 30.5% of the subjects.

Baseline data. Statistical analysis demonstrated that the severity of disease as determined by the number of gingival pockets (≥5 mm) was inversely associated with the number of pathogens detected (presence of one, two, or three periodontal pathogens) (P = 0.001) independent of supragingival plaque scores. Subjects who harbored only a single pathogen had a mean number of 90 (±4) pockets (≥5 mm) present, whereas subjects with two detectable pathogens had a mean number of 79 (±3) pockets present and subjects with all three periopathogens present had a mean number of 68 (±4) pockets present. This difference, however, was not observed in terms of whole-mouth gingival probing pocket depth. The detection of two or three pathogens was associated with lower whole-mouth plaque ($P < 0.001$) and gingival bleeding (P = 0.036) scores compared to clinical data from subjects who harbored only a single pathogen (Table 3). When we compared the differences in the local gingival parameters where microbiological sampling was done, we observed similar differences, but they were not statistically significant.

Posttherapy data. Analysis demonstrated a significant reduction in the numbers of subjects harboring *P. gingivalis* ($P <$ 0.001) or *T. forsythia* (P = 0.043) at 2 months after periodontal therapy compared to the pretreatment data. There was no significant difference between the number of subjects before treatment and at 2 months after treatment with respect to the numbers or proportions that harbored *A. actinomycetemcomitans*. However, all three periodontal pathogens were detected less frequently at 6 months posttreatment compared to the pretreatment prevalence data (Table 2).

TABLE 3. Mean full-mouth bleeding scores and full-mouth subgingival plaque scores in subjects harboring one, two, or all three periodontal pathogens

No. of pathogens detected	Full-mouth bleeding score			Full-mouth subgingival plaque score		
	n (%)	Mean	SE	n (%)	Mean	SE
1	28 (27)	69.6	2.8	28 (27)	67.7	2.8
2	45 (43)	59.9	2.2	45 (43)	52.1	3.3
3	32 (30)	63.9	3.2	32 (30)	57.6	3.8

TABLE 4. Mean full-mouth probing depths and numbers of pockets (≥5 mm) present in subjects harboring one, two, or all three periodontal pathogens

No. of pathogens detected	Full-mouth PPD (mm)			No. of pockets ≥5 mm		
	n (%)	Mean	SE	n (%)	Mean	SE
1	25 (29)	3.4	0.1	25 (29)	36	3.0
2	36 (42)	3.2	0.1	36 (42)	23	3.0
3	25 (29)	3.1	0.1	25 (29)	23	3.0

The presence of more than one pathogen in an individual subject was common, with 72% of the 107 subjects harboring a combination of any two, or all three, of the periodontal pathogens before treatment commenced. After treatment, 65.6% and 40.4% of the subjects at 2 and 6 months posttreatment harbored more than one periodontal pathogen, with significantly fewer subjects harboring more than one pathogen at 6 months posttreatment compared to the baseline data (P = 0.030). Recolonization of periodontal pockets with these pathogens did occur in some of the patients at 6 months posttreatment. Recolonization with *T. forsythia* was most frequently seen; of the 23 patients in whom *T. forsythia* could be detected at baseline but not at 2 months posttreatment, 11 (47.8%) were subsequently positive for *T. forsythia* at 6 months posttreatment. Recolonization of the periodontal pockets with *P. gingivalis* and *A. actinomycetemcomitans* at 6 months occurred in 35.5% and 21.1% of the patients who were negative for these pathogens at 2 months posttherapy.

Statistical analysis confirmed that at 2 months posttherapy, the increasing presence of any of the three periodontal pathogens was associated with a lower PPD (P = 0.04) and number of pockets (≥5 mm) present (P = 0.015), compared to clinical data obtained from subjects who did not harbor any of these pathogens, independent of whole-mouth plaque scores (Table 4). At 6 months posttreatment, this difference was no longer evident, and no association between the presence of individual or combined periodontal pathogens and increased disease severity could be detected. To determine if there was a progression in the severity of the disease between 2 and 6 months, analysis of changes in CAL at each visit was carried out. Using loss of ≥2 mm of periodontal attachment as clinically relevant disease progression, there was no difference in the progression rate of the disease in subjects who harbored any of the three pathogens either individually or in specific combinations.

DISCUSSION

Before periodontal treatment commenced, *T. forsythia* and *P. gingivalis* were detectable in the majority of the subjects (81.3% and 77.6%, respectively). In contrast, *A. actinomycetemcomitans* was harbored in fewer than half (46.7%) of these subjects. Several studies have used a single-round PCR method to detect the presence of these periodontal pathogens. Data from previous molecularly based studies demonstrated that *T.*

forsythia was detectable in 64% to 88% (4, 15, 18, 21), *P. gingivalis* in 20% to 79% (4, 15, 17–19), and *A. actinomycetemcomitans* in 20% to 4% (4, 13, 18, 19) of patients with periodontitis. The high prevalence of periodontal pathogens demonstrated in our study may be due to the nested PCR methodology used, which allows the detection of only 10 cells/ml of these pathogens (9), a level beyond that possible in some of these previous studies. The control of periodontal diseases relies upon subgingival debridement, and this study has demonstrated that a reduction in the presence of these periodontopathogenic bacteria in subgingival plaque occurs posttherapy, in the absence of antibiotic use. However, recolonization of periodontal pockets with pathogens did occur in some of the patients at 6 months posttreatment.

Interestingly, before periodontal therapy commenced, subjects who harbored only one of the periodontal pathogens had a greater number of periodontal pockets (>5 mm) present (90 ± 4 pockets) than those subjects who harbored two (79 ± 3 pockets) or all three (68 ± 4 pockets) of these periodontal pathogens. This greater level of disease in subjects who harbored only a single pathogen was also demonstrated at 2 months posttherapy, with those subjects who harbored only a single pathogen having greater PPD and numbers of pockets present. The reason for this observation is unclear; however, the microbial community present in subgingival plaque is complex, and bacterial interactions, whether positive (synergism or commensalism) or negative (antagonism), are likely to play an important role in the development and maintenance of the members of this community (16) and thereby influence the severity of periodontal disease. Alexander (1) proposed that the ability to maintain homeostasis within a microbial community increases with species diversity. Previous in vitro studies have investigated potential anatagonisic microbial interactions (5, 6, 11). Bostanci et al. (5) demonstrated that *P. gingivalis* was able to antagonize the ability of other bacterial species (including periodontal pathogens) to induce production of the proinflammatory cytokine interleukin-1α (IL-1α). Other work has shown that *P. gingivalis* was able to antagonize the ability of *Campylobacter rectus* to induce production of IL-6 and IL-8 (6). Johansson et al. (11) demonstrated that several periodontal bacteria are able to inhibit the activity of the *A. actinomycetemcomitans* leukotoxin; indeed, *P. gingivalis* exhibited the strongest inhibition of *A. actinomycetemcomitans* leukotoxicity. These previous studies have demonstrated that both cytokine production and the activity of virulence determinants can be reduced when the producer organisms are part of a mixed microbial community. If polymicrobial infections are able to moderate the immune response, then it is possible that a reduction in cytokine levels combined with an inhibition of virulence factors may reduce the severity of the disease. Conversely, if a patient's microbiota is less diverse, the species present may not be subjected to the same degree of antagonistic interactions and may therefore be able to promote a greater inflammatory response leading to a more severe clinical outcome. These data suggest that a reduction in the number of species present may be important in moderating the severity of periodontal diseases. Further studies are required to investigate if other microbial combinations also affect the clinical severity of periodontal diseases and also to determine the precise nature of the antagonistic interactions involved.

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