Occurrence of *Porphyromonas gingivalis, Bacteroides forsythus,* and *Treponema denticola* in Periodontally Healthy and Diseased Subjects as Determined by an ELISA Technique

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THE AIM OF THIS STUDY WAS TO ASSESS, by means of an ELISA technique, the occurrence of 3 putative periodontopathogens, *Porphyromonas gingivalis, Bacteroides forsythus,* and *Treponema denticola,* in 3 clinically-defined adult periodontal conditions. Thirty systemically-healthy subjects were selected and grouped into 3 categories according to their periodontal health: 1) 10 periodontitis subjects (PS), having moderate adult chronic periodontitis; 2) 10 untreated gingivitis subjects (UGS), exhibiting no signs of periodontal destruction but presenting with clinical signs of mild gingivitis; and, 3) 10 treated gingivitis subjects (TGS), having the same clinical status as UGS, but who received a thorough prophylaxis treatment within the past 7 to 14 days prior to the baseline examination. A total of 60 samples were collected subgingivally from the six Ramfjord teeth per subject in each group and ELISA analysis was carried out to give a semiquantitative estimate of *P. gingivalis, B. forsythus,* and *T. denticola.* The immunologic detection method suggested the presence of antigens of *P. gingivalis, B. forsythus,* and *T. denticola* in subjects from each of the 3 groups. When a global analysis for the 3 disease groups was performed at one time, statistically significant differences were found among the ELISA scores of the 3 bacterial species. For example, comparisons of the ELISA scores showed that the concentrations of *P. gingivalis* differed significantly when comparing TGS to UGS and PS, but not when examining UGS/PS. The ELISA scores for *B. forsythus* were significantly different between TGS and PS. Mean concentrations of *T. denticola* were significantly different when comparing PS to TGS or UGS, whereas no difference was found between the latter categories. Within the limited scope of this study, the concentration of antigens detectable from putative periodontopathogens like *P. gingivalis, B. forsythus,* and *T. denticola* differed among the 3 diseased groups, with periodontitis subjects often showing the greatest level of antigens. Thus, it is reasonable to expect that, when using sensitive immunological detection methods, antigens of suspected periodontal pathogens can be found irrespective of the individual's clinical status. However, while detectable in the periodontal sites, the concentrations of these microorganisms are most likely to be above the threshold necessary to induce clinically-significant disease. Studies with larger sample size and standardized antigens are necessary to determine if the groups we found not to differ, were, in fact, different. *J Periodontol 1997;68: 18–23.*

Key Words: *Porphyromonas gingivalis; Bacteroides forsythus; Treponema denticola;* gingivitis/microbiology; periodontitis/microbiology.

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The primary role of bacteria in periodontal inflammation and destruction is well established. Moreover the association between bacterial morphotypes and species to various periodontal conditions has also been well documented.[1-5] In the past several years it has been postulated that specific bacteria could be related to active tissue destruction during periodontal disease.[6-9] On the basis of such a concept, microbiological tests have been proposed to offer the clinician valuable information in the management of periodontal patients.[10,11]

Cultural methods have usually been considered the "gold standard"[10] for assessing the presence of specific bacteria in periodontal pockets, as most studies have relied on cultural analyses of subgingival plaque. However, more sensitive identification methods (i.e., DNA probes, immunological techniques) have been recently used in clinical and microbiological studies. *Porphyromonas gingivalis, Bacteroides forsythus,* and *Treponema denticola* are putative periodontopathogens which have been extensively studied in recent years.[12-16] Since their presence has been associated with severity of disease and attachment loss, *P. gingivalis, B. forsythus,* and *T. denticola* are among those bacterial species which are considered microbiological markers of periodontal disease and the presence of these species can be detected by a quick enzyme reaction-based test.[17] However, recently the direct association between ongoing periodontal destruction and specific bacterial species has been questioned.[18-20] On the basis of such considerations the aim of the present study was to observe, by means of an ELISA technique, the presence of three putative periodontopathogens; namely, *P. gingivalis, B. forsythus,* and *T. denticola,* in 3 clinically-defined adult periodontal conditions.

MATERIALS AND METHODS

Subjects

Thirty systemically healthy subjects (11 males and 19 females, ages 27 to 71 years) who had not taken antibiotics in the previous 6 months who were referred to the Dental Clinic University of Rome La Sapienza were selected and grouped in 3 categories, representing defined periodontal conditions: 1) periodontitis (PS) (n = 10) showing radiographic evidence of bone loss (≥ 30%) and clinical signs of moderate adult chronic periodontitis (i.e., true pocketing, evidence of attachment loss and bleeding on probing); 2) untreated gingivitis (UGS) (n = 10) having no attachment loss or radiographic evidence of bone loss at any sites, but presenting with moderate plaque accumulation (PI score = 1 or 2) and clinical signs of mild gingivitis (i.e., ≥ 30% of sites with bleeding on probing); or 3) treated gingivitis (TGS) (n = 10) with same clinical conditions as UGS, but who received prophylaxis treatment within 7 to 14 days prior to baseline examination together with instructions on proper brushing and dental flossing given during the month prior to the commencement of the study.

Six distal-lingual sites on the six Ramfjord teeth[21] were selected in each individual for a total of 60 sites in each clinical category (total: 180 sites). The following clinical parameters were recorded: probing depth (PD), plaque index (PI),[22] and gingival bleeding index (GBI),[23] all assessed with a Michigan "0" probe. After removal of supragingival plaque with a cotton pellet, subgingival plaque was collected using a sterile Gracey curet and deposited onto a paper support which was express mailed to the Immunology Laboratory, The University of Michigan, School of Dentistry, Ann Arbor, MI for ELISA analysis.

This protocol was approved by the authors' institutional review committee for human subjects and was performed in accordance with the Helsinki Declaration of 1975, as revised in Immunologic analysis for bacterial antigens. The ELISA assay, as described by Van Poperin and Lopatin[14] for detection of microbial species in plaque samples, was used in this study. Highly specific hyperimmune rabbit antibodies to *P. gingivalis, T. denticola,* and *B. forsythus* were prepared as described by Bretz et al.[24] Antibodies bound to target bacterial cells were stained with alkaline phosphatase-conjugated anti-rabbit immunoglobulins. The immune complex was detected and quantitated after incubation with a BCIP/NBT phosphatase substrate system.[§] Both positive and negative controls were included for the ELISA test. The reference strains (positive controls) for these microorganisms were all ATCC strains: *P. gingivalis* strain ATCC 33277; *T. denticola* strain ATCC 35405; and *B. forsythus* strain ATCC 43037. All antisera were tested against a panel of related and unrelated species to ensure specificity. These antisera have been compared to DNA probes.[25] The dilution of the antisera was also chosen to minimize cross-reaction.[24] There was minimal detectable cross-reactivity with unrelated species when the antisera were used in our ELISA system. In order to simplify reporting of the relative amount of each microbial species, ELISA results were scored as $0 = \leq 5 \times 10^4$; $1 = 5 \times 10^4 < 1 \times 10^5$; $2 = < 5 \times 10^5$, or $3 = \geq 5 \times 10^5$ bacteria per sample (b/s).

Statistical Analysis

Patients were used as the statistical unit. When all three groups were compared, Kruskal-Wallis test was employed to detect significant differences for PI, while analysis of variance (ANOVA) was applied to recognize statistically significant differences in PD measurements and GBI scores. *Post hoc* two-group comparisons of clinical indices were assessed with the Bonferroni-corrected Mann-Whitney U test (PI) and Student *t*-test for unpaired samples (GBI and PD).

[§]Kirkegaard and Perry Laboratories, Gaithersburg, MD.

Table 1. Clinical Measurements of the 3 Groups

Measurement	TGS	UGS	PS
Median PI score	0	1	2
% GBI positive sites	10	47	90
Mean PD (mm)	2.0	2.2	4.6

TGS = treated gingivitis subjects; UGS = untreated gingivitis subjects; PS = periodontitis subjects.
PI = plaque index; GBI = gingival bleeding index; PD = probing depth.

Differences in detection of *P. gingivalis, B. forsythus,* and *T. denticola* antigens among the 3 categories, considered as a whole, were evaluated by the Kruskal-Wallis test. The Bonferroni-corrected Mann-Whitney U test was used in 2-group comparisons to evaluate differences for each microbial species, examining two subject groups at a time.

RESULTS

Clinical Findings

Table 1 reports the clinical status for each subject category. Statistical analysis of clinical data showed significant differences between categories. Statistical comparisons between two subject categories at a time are reported in Table 2. Mean PI differed significantly in the 3 groups ranging from 0.28 for TGS to 1.71 for PS. GBI differed significantly as well. Ten percent of the sites in TGS group had a positive GBI score, whereas in UGS and PS, 47% and 90%, respectively, of total sites were positive. Mean probing depths were 2.0 mm, 2.2 mm, and 4.6 mm for TGS, UGS, and PS, respectively. As expected, PD from UGS did not differ significantly from that of TGS. In contrast, there was a significant difference between PS on one side, and TGS and UGS on the other.

Detection of Bacterial Antigens

Prevalence of *P. gingivalis, B. forsythus,* and *T. denticola* antigens reached 100% in all 3 categories. Median individual ELISA scores for the 3 groups are reported in Table 3. *P. gingivalis* had a median score of 2.2 in TGS, while it reached 3.0 in both UGS and PS. ELISA scores for *B. forsythus* and *T. denticola* were 1.0, 1.5; 1.5, 2.3; and 2.5, 3.0 in TGS, UGS, and PS, respectively. When the median scores were compared simultaneously, statistically significant differences existed among the 3 periodontal status categories for all 3 bacterial species (Table 4). Two-group comparisons showed, however, that *P. gingivalis* scores were significantly different only when comparing TGS with UGS and TGS with PS. Median scores of *B. forsythus* differed significantly only between TGS and PS, whereas values of *T. denticola* did not vary significantly between TGS and UGS.

Table 3. Median ELISA Scores of the Subject Groups

	TGS	UGS	PS
Porphyromonas gingivalis	2.2	3.0	3.0
Bacteroides forsythus	1.0	1.5	2.5
Treponema denticola	1.5	2.3	3.0

TGS = treated gingivitis subjects; UGS = untreated gingivitis subjects; PS = periodontitis subjects.

Table 4. Statistical Analyses of Differences Between Median ELISA Scores in the Subject Groups

	Difference			
	Considered as a Whole	TGS/UGS	TGS/PS	UGS/PS
P. gingivalis	$P < 0.001$	$P < 0.05$	$P < 0.01$	NS
B. forsythus	$P < 0.05$	NS	$P < 0.05$	NS
T. denticola	$P < 0.001$	NS	$P < 0.001$	$P < 0.01$

TGS = treated gingivitis subjects; UGS = untreated gingivitis subjects; PS = periodontitis subjects.
NS = not significant.

DISCUSSION

The three groups in this study differed significantly from one another in terms of clinical conditions, as assessed by PI, GBI, and PD measurements. Obviously, the TGS group did not differ from the UGS group when PD data were considered and this suggests that the gingival disease may have persisted or had not fully resolved in the short time after scaling (Tables 1 and 2). However, from the microbiological standpoint, *P. gingivalis, B. forsythus,* and *T. denticola* antigens were detectable in all subjects in all clinical categories. While the prevalence was similar in all groups, concentrations of the studied bacteria varied significantly when the data regarding TGS, UGS, and PS were analyzed simultaneously. These data confirm previous studies that indicated plaque concentra-

Table 2. Statistical Analyses of Differences Between Clinical Parameters of the Subject Groups*

Parameters	Considered as a Whole	TGS/UGS	TGS/PS	UGS/PS
PI score	$P < 0.001$; S	$P < 0.05$; S	$P < 0.01$; S	$P < 0.01$; S
% GBI positive sites	$P < 0.005$; S	$P < 0.05$; S	$P < 0.01$; S	$P < 0.05$; S
Mean PD	$P < 0.01$; S	$P > 0.05$; NS	$P < 0.01$; S	$P < 0.05$; S

*Analyses are carried out comparing all three categories first, and then considering two groups at a time. S = significant; NS = not significant.
TGS = treated gingivitis subjects; UGS = untreated gingivitis subjects; PS = periodontitis subjects.
PI = plaque index; GBI = gingival bleeding index; PD = probing depth.

Table 5. Number and Percentage of Positive Sites for *P. gingivalis, B. forsythus,* and *T. denticola* at Different ELISA Score Thresholds

ELISA threshold	TGS		UGS		PS	
Pg > 0	57	95.0%	57	95.0%	59	98.3%
Pg > 1	47	78.3%	53	88.3%	56	93.3%
Pg > 2	25	41.6%	45	75.0%	53	88.3%
Bf > 0	34	56.6%	54	90.0%	44	73.3%
Bf > 1	18	30.0%	29	48.3%	42	70.0%
Bf > 2	11	18.3%	19	31.6%	37	61.6%
Td > 0	47	78.3%	51	85.0%	59	98.3%
Td > 1	29	48.3%	41	68.3%	57	95.0%
Td > 2	6	10.0%	24	40.0%	49	81.6%

TGS = treated gingivitis subjects; UGS = untreated gingivitis subjects; PS = periodontitis subjects.
Pg = Porphyromonas gingivalis; *Bf = Bacteroides forsythus*; *Td = Treponema denticola.*

tions of *P. gingivalis, B. forsythus,* and *T. denticola* differ with varying periodontal conditions.[12–16] However, *post hoc* two-group analyses showed no difference between TGS and UGS for antigens of *B. forsythus* and *T. denticola,* whereas values for antigens of *P. gingivalis* and *B. forsythus* were not significantly different between UGS and PS (Table 4). The 3 microorganisms were always present, not only in subjects diagnosed with periodontitis, but also in those who presented with clinical signs of mild inflammation and no periodontal destruction. Moreover, *P. gingivalis, B. forsythus,* and *T. denticola* were also detected in those subjects who had previously undergone a meticulous professional prophylaxis. Periodontally healthy subjects were included in this study to serve as negative controls and exhibited a median PI score of 0.0 and were GBI-positive only in 10% of the sites. The fact that *P. gingivalis, B. forsythus,* and *T. denticola* were present in all individuals included in the TGS group suggests that these microorganisms may be normal components, although at rather low concentrations, of the oral microflora or show resistance to common mechanical debridement techniques. In fact, full resolution of gingivitis may not have taken place in the short time after scaling in the TGS group.

Particular attention must be paid to data regarding *P. gingivalis,* a microorganism considered a classic disease marker in periodontal diseases.[26,27] The prevalence of this bacterium in healthy individuals and in gingivitis patients is reported to be very low in adults.[28–30] When we analyzed data in a pairwise manner, we found that the median ELISA scores of *P. gingivalis* did not differ significantly between UGS and PS. Moreover, *P. gingivalis* was found in 41% of the sites in the TGS and in 75% of the sites in the UGS group (Table 5). In the former clinical category, 9 out of 10 subjects had a *P. gingivalis* ELISA score of 3 in at least one site.

Our findings are quite different from previous reports of *P. gingivalis* colonization.[28,30–33] However, those studies used cultural techniques to enumerate microorganisms in subgingival plaque. This might account for the apparent discrepancy existing between our findings and those from other studies.[28,30–33] Some investigators have suggested the existence of a strict relationship between the detection of *P. gingivalis* and the risk of periodontal disease at a given site.[34,35] The high prevalence of *P. gingivalis* even in healthy subjects could be due to the detection technique employed in the present study, although the possibility of false positives due to cross-reactions cannot be ruled out by our study. A second, confirmatory technique would have to be applied to our samples to rule out cross reactivity. The sensitivity of the ELISA assay used in this study may help explain the difference between our data and those reported in literature. In a study comparing culturing, DNA probes, immunofluorescence, and ELISA, Loesche et al.[25] observed the lowest prevalence values with the cultural technique whereas the highest values were obtained with the immunological and DNA probe techniques. In particular, prevalences of *P. gingivalis* and *B. forsythus,* as assessed by ELISA, reached 89% and 64%, respectively. In contrast, prevalence values for *P. gingivalis* and *B. forsythus* in the same plaque samples were 43% and 25% when culturing was employed. Racial and environmental factors may also account for the high prevalence of *P. gingivalis, B. forsythus,* and *T. denticola.* Dahlen et al.[36] and van Steenbergen et al.[37] have isolated *P. gingivalis* in Kenyan and Indonesian populations in 70% and 87% of the subjects examined, respectively. Dahlen et al.[38] reported that "deep periodontal pockets were not a prerequisite ecological environment for *P. gingivalis* establishment." Although the subjects we examined were all Caucasians, and not to be compared to the above mentioned populations, racial and environmental factors cannot be completely overruled when considering the high prevalence of *P. gingivalis.*

The existence of strains of *P. gingivalis* with different virulence properties as found by Neiders et al.[39] and Smalley et al.[40] could explain why *P. gingivalis* is found extensively in periodontally healthy patients without any clinical sign of inflammation in our study. The small size of the 3 subject groups may have also affected the results

of our study. The lack of difference in the 2-group comparisons of UGS versus PS for *P. gingivalis,* TGS versus UGS, UGS versus PS for *B. forsythus* and TGS versus UGS for *T. denticola* is probably due to the small sample size. Thus, we cannot exclude the possibility that these results reflect a type II statistical error. Nevertheless, it is striking that *P. gingivalis, B. forsythus,* and *T. denticola*—3 putative periopathogens—were found in all subjects enrolled in our study, including individuals subjected to thorough mechanical prophylaxis measures. It may be that TGS subjects experienced recurrent colonization of the 3 bacterial species from extra-dental locations like tonsils and the dorsum of the tongue, which have been shown to act as potential reservoirs for putative periopathogens.[41] However, we do not have any data in this study to conclude, nor do we expect, that the microorganisms were eradicated by the professionally-delivered cleaning. Since presence of or simple infection with a pathogenic microorganism does not imply disease, concentration is likely to be the key factor involved in disease onset and progression.[42] Exceeding a threshold concentration would be expected to result in disease. In conclusion, all 30 patients in our study and the majority of sites in UGS and PS (and a discrete portion in TGS) contained detectable levels of *P. gingivalis, B. forsythus,* and *T. denticola* despite fairly different clinical situations. Bearing in mind the limited scope of the present work, it is reasonable to conclude that relying only on the mere detection of one or more periopathogens to assess the status of periodontal disease might be misleading if other disease markers are not included in the evaluation of periodontal patients.[42]

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