Prevalence of *Treponema denticola* and *Porphyromonas gingivalis* in plaque from periodontally-healthy and periodontally-diseased sites

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Summary. Intracrevicular plaque from periodontally-healthy individuals who had refrained from oral hygiene measures for 24 h prior to sampling, and subgingival plaque from diseased sites of patients with chronic periodontitis were screened by ELISA for the presence of *Porphyromonas gingivalis* and *Treponema denticola*. The samples were also subjected to the PerioScan test to detect the presence of enzymes capable of degrading N-benzoyl-DL-arginine-2-naphthylamide (BANA). Of the 141 samples from periodontally-healthy sites, 73 % contained *T. denticola* antigens and 78 % *P. gingivalis* antigens, compared to 43 % and 59 %, respectively, in plaque samples from the 159 diseased sites. A positive reaction in the PerioScan test was obtained in 89 % of plaque samples from diseased sites and in 60 % of those from healthy sites. The correlation between the results of the two assays was poor in the case of intracrevicular plaque from healthy sites. However, with plaque samples from diseased sites, the results of the PerioScan test showed very strong correlation with those obtained with the ELISA, suggesting that the former may be a useful, rapid means of indicating the presence of *T. denticola* and *P. gingivalis* in such plaque samples.

Introduction

Several bacterial species are thought to have an aetiological role in the various forms of periodontal disease. These include *Porphyromonas gingivalis*,^{1,2} *Bacteroides forsythus*,³ *Actinobacillus actinomycetem-comitans*,⁴ *Wolinella recta*⁵ and *Treponema denticola*.⁶ Whether these organisms are members of the indigenous flora or are exogenous oral pathogens is the subject of continuing debate, and resolution would be aided by more information regarding the prevalence of these organisms in both healthy and diseased sites.

Considerable interest has developed in methods of detecting periodontopathogens in plaque samples. Available techniques include the use of cultural procedures, microscopy,⁷ antibodies⁸ and DNA probes.⁹ An alternative approach involving the detection of enzymes produced by the organisms of interest has also been suggested.¹⁰ One particular enzyme, with a trypsin-like activity, has attracted much attention because it is produced by only a few members of the cultivable oral microflora, namely *P. gingivalis*, *B. forsythus*, *T. denticola* and a *Capnocytophaga* sp.^{10–13} The presence of this enzyme in, e.g., a plaque sample can be established by the hydrolysis of a synthetic trypsin substrate, N-benzoyl-DL-arginine-2-naphthyl-

amide (BANA), releasing β -naphthylamine. The β -naphthylamine produced can be detected by its reaction with Evans black dye which results in a blue-black product. This is used as the basis of a test (PerioScan, Oral-B Laboratories, USA) for the detection of BANA-hydrolysing bacteria in plaque samples.

The purpose of this investigation was to screen intracrevicular marginal and subgingival plaque samples from periodontally-healthy and periodontally-diseased subjects, respectively, for the presence of two disease-associated organisms, *P. gingivalis* and *T. denticola*, with both an ELISA assay and the PerioScan test. Since levels of the target organisms might be expected to be low in periodontally-healthy individuals, plaque was allowed to accumulate in these subjects for 24 h before sampling. Thus it was anticipated that this amplification *in vivo* would permit small numbers of target organisms, which would otherwise escape detection, to multiply to detectable levels without influencing the clinical status of the marginal tissues.

Materials and methods

Subjects

Forty-six subjects (32 females and 14 males) were

selected from departmental staff and from patients attending the Department of Periodontology of the Eastman Dental Hospital. Their ages were 18–59 years mean 35.5 years. None had taken any antibiotics or had periodontal therapy during the previous 3 months. The subjects were divided into two groups depending on their periodontal status. A “healthy” group was represented by 18 members of the clinical staff with very good oral hygiene, clinically healthy gingiva and no periodontal attachment loss. The “diseased” group consisted of 28 adult patients presenting with chronic inflammatory periodontal disease (CIPD) with significant degrees of attachment loss and corresponding radiographic signs of bone loss.

Plaque samples

Individuals in the healthy group were asked to refrain from oral hygiene measures for 24 h before sampling. This allowed plaque to accumulate with an increase in the number of any target organisms initially present. Intracrevicular plaque was then sampled from Ramfjord teeth, i.e., upper right first molar and first incisor, lower left first incisor and first molar, upper left first premolar and lower right first incisor. In the diseased group, supragingival plaque was carefully removed with hand scalers at each test (diseased) site and subgingival plaque was sampled with a fine curette. Samples were taken from at least six sites in each subject.

Clinical measurements

Probing depth was measured with a William's probe from the gingival margin to the base of the pocket. The papillary bleeding index was determined as described by Saxer and Muhlemann.¹⁴

Assay for BANA-hydrolysing activity

The PerioScan (Oral-B Ltd) test procedure was performed according to the manufacturer's recommended procedure. Each plaque sample was transferred on to the surface of a BANA-containing strip mounted on a plastic card. A parallel strip containing Evans fast black dye was moistened with distilled water and the card was then folded so that the two strips were in contact. The card was then incubated at 55°C for 15 min. After incubation, the card was unfolded and examined for the presence (positive) or absence (negative) of a blue colour on the dye-containing strip.

ELISA

Following the evaluation of the PerioScan card for BANA reactivity, the cards were re-folded and posted to the Immunology Laboratory of the School of Dentistry, University of Michigan for ELISA analysis. The lower portion of the card was cut in half, splitting each plaque specimen into two portions. Each strip

was then immersed in Tris-buffered saline (TBS; 0.05 M NaCl, 10 mM Tris, pH 7.4) containing non-fat dry milk 0.5% (BLOTTO) for 30 min to block any subsequent non-specific binding. The strips were then incubated with the appropriate hyperimmune rabbit polyclonal antimicrobial antibody¹⁵ diluted in TBS containing Tween-20 0.5% (TBS-T) for 1 h at room temperature. After three 5-min washes in TBS-T, goat anti-rabbit immunoglobulin G (heavy and light chains) conjugated to alkaline phosphatase (BioRad) diluted in TBS-T containing BLOTTO 0.5% was applied and incubated for 1 h. After the final three 5-min washes, BCIP-NBT substrate solution (Kirkegaard and Perry Laboratories) was applied and colour development was allowed to proceed to its maximum. Before processing, solutions of pure bacterial suspensions were applied to the strips to serve as reference standards. The results obtained with plaque samples were compared to those of the standards and were scored on a scale of 0–3, where 0 was negative, 1 was detectable, 3 was the highest intensity and 2 was intermediate. This procedure was found to detect $c. 5 \times 10^4$ cfu and was maximal at $c. 1 \times 10^6$ cfu. In practice, scores of 0 and 1 were considered to be negative and scores of 2 and 3, positive.

The antibodies used in this study were highly specific for their respective micro-organisms. All antibodies were tested by ELISA against an extensive panel of oral micro-organisms (clinical isolates and ATCC strains) to demonstrate their specificity. When appropriate, exhaustive absorption procedures were performed to remove cross-reactivity. The sensitivity and specificity of these antibodies, compared to DNA probes and cultural analysis, have been reported previously.^{16,17}

The reproducibility of ELISA after long-term storage of the PerioScan cards was investigated. It was found that once pure cultures or plaque samples had been applied to the cards and dried, storage for as long as a year had no significant effect on the detectability of the specific antigens. Tests of stability at ambient temperatures suggested that the integrity of the experimental samples was not compromised during transport to the USA.

Statistical analysis

The results of the PerioScan test were compared with those of the ELISA (which was taken to be the standard) by means of a contingency table. The sensitivity was defined as the number of true positives divided by the sum of the true positives and false negatives. The specificity was defined as the number of true negatives divided by the sum of the false positives and true negatives.

Results

The clinical data pertaining to the 300 sites examined and sampled are shown in table I; 159 were diagnosed

Table I. Clinical data for the healthy and diseased sites

Measurement		Healthy sites	Diseased sites
Papillary bleeding score	range	0-3	0-4
	mean	0.52	2.96
	mode	0	3
Probing depth (mm)	range	1-5	1-10
	mean	1.7	5.2
	mode	2	5

as diseased and 141 as clinically healthy. As shown in table II, *P. gingivalis* was detected by ELISA more often in the healthy sites (78%) than in the diseased sites (59%) and this difference was statistically significant ($p < 0.001$, z test of the difference between two unpaired proportions). Similarly, the frequency of detection of *T. denticola* by ELISA was greater in the healthy sites (73%) than in the diseased sites (43%), this difference was also statistically significant ($p < 0.001$, z test of the difference between two unpaired proportions). A much greater proportion of healthy sites (61%) than diseased sites (40%) harboured both organisms whilst in only 11% of the healthy sites, compared to 38% of the diseased sites, was neither of the organisms detected.

The PerioScan test was positive with 142 (89%) of the subgingival plaque samples from the diseased sites compared to only 85 (60%) of the intracrevicular margin samples from the non-diseased sites (table III). The PerioScan test was positive with 97 (99%) of 98 diseased site plaque samples in which either *P. gingivalis* or *T. denticola* had been detected by ELISA and in 63 (98%) of 64 plaque samples in which both organisms were present. However, a positive PerioScan reaction was also obtained with 45 (74%) of 61 samples in which, on the basis of the ELISA test, neither of these organisms was present. In the healthy sites, the PerioScan test was positive in 73 (58%) of 126 sites shown by ELISA to contain either *P. gingivalis* or *T. denticola* and in 57 (66%) of 86 sites containing both organisms. In contrast to the results obtained from the diseased sites, 53 (42%) of 126 sites containing either *P. gingivalis* or *T. denticola* failed to give a positive PerioScan reaction.

The sensitivity and specificity of the PerioScan test, with the ELISA as a standard, are shown in table IV where it can be seen that the test had a high sensitivity

Table III. Results obtained following PerioScan-testing of the plaque samples. The presence of *P. gingivalis* and *T. denticola* in the plaque samples is also shown, these being determined by an ELISA

PerioScan result	ELISA result	Number of samples from	
		Healthy sites	Diseased sites
+	...	85	142
+	Pg or Td +	73	97
+	Pg and Td -	12	45
+	Pg and Td +	57	63
+	Pg +	70	92
+	Td +	60	67
-	Pg or Td +	53	1
-	Pg and Td -	3	16

Pg, *P. gingivalis*; Td, *T. denticola*.

Table IV. Sensitivity and specificity of the PerioScan test with an ELISA for *T. denticola* and *P. gingivalis* as standard

Sites	Sensitivity (%)	Specificity (%)
Healthy	58	80
Diseased	99	26
All	76	25

(99%) when used to screen subgingival plaque samples from the diseased sites, but a lower sensitivity (58%) when applied to intracrevicular margin plaque samples from healthy sites. However, the specificity was higher in the case of intracrevicular margin plaques (80%) than in that of the subgingival plaques (26%).

In both healthy and diseased sites, a positive PerioScan result showed a greater degree of correlation with the presence of *P. gingivalis* than with the presence of *T. denticola*. In healthy sites, 82% of the PerioScan positive plaques contained *P. gingivalis* and 71% contained *T. denticola*, whereas in diseased sites, these proportions were rather lower at 65% and 47%, respectively.

Discussion

The results of the present study have revealed a high prevalence of *T. denticola* and *P. gingivalis* in the plaque associated with both healthy and diseased sites. Indeed, these organisms were detected more frequently

Table II. Prevalence of *P. gingivalis* and *T. denticola* as detected by ELISA in subgingival plaque from diseased sites and in intracrevicular margin plaque from healthy sites

Type of site	n	Number (%) of sites with				
		<i>T. denticola</i>	<i>P. gingivalis</i>	both	either	neither
Healthy	141	103 (73)	110 (78)	86 (61)	126 (89)	15 (11)
Diseased	159	68 (43)	94 (59)	64 (40)	98 (62)	61 (38)

in relation to clinically healthy gingival margins than in subgingival plaque from individuals with periodontitis.

There are conflicting reports of the prevalence of *T. denticola* in plaque samples from periodontally-healthy sites. With an ELISA test, Simonson *et al.*⁶ reported similar results to those of the present study in that each of the 37 plaque samples obtained from periodontally-healthy interproximal sites contained a *T. denticola* antigen. This was endorsed by Watson *et al.*¹⁸ who detected *T. denticola* by ELISA in "subgingival" plaque samples from most (79%) of 157 periodontally-healthy children. In contrast, Bretz *et al.*¹⁹ could not detect *T. denticola* in any of 19 "subgingival" plaque samples from periodontally-healthy individuals. The disparate results between these investigations could reflect differences in assay sensitivity or specificity or both.

Our finding that *T. denticola* was detected in only 43% of diseased sites but in 73% of healthy sites is notable as *T. denticola* has been detected in 100%⁶ and 63%¹⁹ of subgingival plaque samples from sites with moderate or advanced periodontitis. In this investigation, a high prevalence of *P. gingivalis* was found in both subject groups, this being greater in healthy sites (78%) than in diseased sites (59%). Other ELISA-based studies on healthy sites have reported similarly high frequencies of detection.¹⁸ However, culture studies have revealed an isolation rate of c.10% for *P. gingivalis* from healthy sites.²⁰ Zambon *et al.*⁸ were unable to culture *P. gingivalis* from periodontally-healthy adults, but 30% proved to be positive by immunofluorescence microscopy. With a DNA probe (detection level 10^3 cells), *P. gingivalis* was found in 20.4% and 41.8% of 59 "subgingival" plaque samples from periodontally-healthy children with a Modified Plaque Index of 0 and 1, respectively.²¹

Two factors may account for the unexpected finding of a high prevalence of *T. denticola* and *P. gingivalis* in healthy sites in the present study. Firstly, the healthy subjects refrained from oral hygiene measures for 24 h before sampling, allowing the small numbers of *T. denticola* and *P. gingivalis* which may have been present initially to proliferate to levels detectable by the highly sensitive ELISA. Secondly, as many workers have not employed the more sensitive techniques involving antigen detection or DNA probes, the prevalence of these organisms in healthy sites may have been under-reported.

A positive PerioScan reaction indicates the presence of BANA-hydrolysing enzymes in the subgingival plaque sample. Studies have shown that the most likely sources of such enzymes are members of the microflora such as *P. gingivalis*, *T. denticola* and *B. forsythus* that are capable of producing a trypsin-like enzyme.^{10,13} Therefore, it has been suggested that the PerioScan test may be a simple, rapid method for detecting the presence of these three important periodontopathogenic bacteria in plaque samples.

In this investigation, 99% of plaque samples from

diseased sites shown by ELISA to contain either *T. denticola* or *P. gingivalis* also gave positive results in the PerioScan test. However, neither *T. denticola* nor *P. gingivalis* could be detected by ELISA in 32% of samples positive by the PerioScan test. This may be attributable to the presence of *B. forsythus* or other, as yet uncultivated, plaque organisms capable of hydrolysing BANA. Unfortunately, at the time of the study, no antibody or DNA probes to test for the presence of *B. forsythus* in the samples were available and cultural methods were deemed unreliable.²² An alternative explanation is that the PerioScan test is more sensitive than the ELISA, but Loesche *et al.*¹³ reported that the PerioScan test could detect c. 10^6 cfu of *T. denticola* and 10^5 cfu of *P. gingivalis* whereas the ELISA was able to detect c. 5×10^4 cfu of either organism. The laboratory strains of the target organisms used by Loesche may have had fewer copies of the trypsin-like enzyme than the strains present in freshly harvested plaque samples. Therefore, when used for detecting target organisms in plaque samples, the PerioScan test may have a sensitivity greater than that suggested by the above study.

The PerioScan test proved to be an extremely sensitive means of detecting the presence of *T. denticola* and *P. gingivalis* in the diseased sites when ELISA was the standard. The low specificity of the test may be the result of an over-estimation of false positives since the samples were not screened for *B. forsythus* by ELISA. Many of these apparent false positives may have been true positives in that they could reflect the presence of *B. forsythus*. Furthermore, subsequent to the present study, Feitosa *et al.*²³ have reported that the specificity of the PerioScan test can be improved by incubation at 37°C rather than at 55°C. The ability of the highly sensitive PerioScan test to detect the presence of these BANA-hydrolysing organisms in subgingival plaque samples from diseased patients suggests that it would be useful in monitoring the effectiveness of treatment regimens aimed at the elimination of these organisms.

In the case of healthy sites, only 58% of the samples determined by ELISA to contain either *T. denticola* or *P. gingivalis* were positive by PerioScan. The failure of the latter to detect the presence of these organisms in the remaining healthy sites may be explained by the much greater sensitivity of the ELISA. The ability of the PerioScan test to give a positive result only when a certain number of the target organisms is present may be useful in discriminating between colonisation by the organism and attainment of a critical, potentially disease-inducing threshold.^{13,18} The question remains as to what this threshold value might be.

Only 14% of healthy sites with neither *T. denticola* nor *P. gingivalis* (on the basis of ELISA) were PerioScan positive—a far lower proportion than in the case of the diseased sites (32%). This implies that, should these false positive reactions be due to the presence of *B. forsythus*, its prevalence was much lower in healthy than in diseased sites, as has been

reported by Lai *et al.*²⁴ With the ELISA as a standard, the sensitivity of the PerioScan test when used on intracrevicular margin plaque samples was low (58 %) but the specificity high (80 %). These results are similar to those of a study of subgingival plaque samples from periodontally-healthy children.¹⁸ The low sensitivity of the PerioScan test compared to the ELISA may be attributable to the lower detection threshold of the latter referred to above.

In conclusion, the present study has shown, by means of a sensitive ELISA, a high prevalence of *T. denticola* and *P. gingivalis* in the gingival crevicular plaque allowed to accumulate for 24 h in periodontally-healthy individuals. These findings support an increasing number of studies demonstrating that

some periodontopathogens may be regarded as members of the normal oral microflora. Also, the PerioScan test, while being far less sensitive than the ELISA in detecting the presence of *P. gingivalis* and *T. denticola* from periodontally-healthy subjects, was almost as sensitive as the ELISA in detecting the presence of these organisms in subgingival plaque samples from patients with chronic periodontitis. This simple test may, therefore, prove to be a reliable, convenient means of monitoring the effectiveness of treatment regimens designed to eradicate these organisms from periodontal pockets.

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