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The effect of incubation temperature on the specificity of the BANA (N-benzoyl-DL-arginine-naphthylamide) test

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The hydrolysis of BANA by subgingival plaque samples is associated with the presence of either *Treponema denticola, Porphyromonas gingivalis,* and/or *Bacteroides forsythus.* A protocol in which pure cultures were incubated for 15 min at 55°C detected about 5×10^5 CFU of *P. gingivalis* and 1×10^6 CFU of *T. denticola.* Clinical studies indicated that the BANA test in this configuration will detect about 10^4 organisms *in vivo* as compared with the 10^5 to 10^6 organisms found with *in vitro* grown cells. The BANA test can be made less sensitive by decreasing the time and/or temperature of incubation, which could improve the specificity of the test. In the present study we determined the incubation parameters that would give optimal specificity when the plaque samples were removed from sites of gingival health. Twenty-six approximal plaque samples were taken from each of 90 clinically healthy subjects and incubated with the BANA substrate on PerioScan cards (Oral-B Laboratories) for 5 and 15 min at 35°, 45°, and 55°C. Subjects were randomly assigned to the various temperatures. Wooden toothpicks were inserted interproximally in all sites anterior to distal of the first molars and then each side of the toothpick was wiped onto the PerioScan card. The specificity of the BANA test relative to clinical health was 96% when the cards were incubated for 5 min at 35°C, but decreased to 50–70% when the cards were incubated for 15 min at 35°C or for 5 and 15 min at 45°C and 55°C. These findings indicate that the specificity of the BANA test can be improved by shortening the incubation period to 5 min and by reducing the incubation temperature to 35°C.

Key words: BANA test; periodontal disease; *Treponema denticola; Porphyromonas gingivalis*

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Treponema denticola, Porphyromonas gingivalis and *Bacteroides forsythus* are the most prominent BANA-hydrolyzing species found in subgingival plaque (6, 9, 14). Because one or more of these species are frequently cited as potential periodontopathogens (2, 7, 12) and all are anaerobes, the ability of a given subgingival plaque to hydrolyze BANA could be a measure of risk for an anaerobic periodontal infection at the sampled site. The PerioScan test is a modification of the BANA test and was developed to provide a rapid, inexpensive chairside method that could provide the clinician with objective information on the presence or absence of these putative periodontopathogens or other BANA-hydrolyzing species in plaque samples (9). This information could help the clinician in deciding the future treatment needs, if any, for a particular site or subject or in assessing the adequacy of treatment already rendered to a particular site in a subject.

T. denticola and *P. gingivalis* are found by immunological reagents to be present in plaque samples taken from periodontally healthy sites in adults (10) and in children (15). This means that most plaque samples would be BANA-positive if the BANA colorimetric assay were performed at maximal sensitivity. The amount of color development in the BANA assay is a function of the amount of enzyme in the sample, the length of incubation, the temperature of incubation, the amount of substrate available and the presence of potentiators or inhibitors in the assay medium. The variable of interest in a periodontal diagnostic test would be the amount of enzyme that could be detected and how this related to the number of organisms in the plaque sample.

We selected 10^6 colony-forming units (CFU) of *T. denticola* and/or *P. gingivalis* as the number of organisms we wanted to detect in a plaque sample, as we assumed that this level would be associated with clinical disease (9). In the laboratory we varied the levels of the BANA substrate and the time and temperature of incubation and found that a 15-min incubation at 55°C would detect 10^6 CFU of *in vitro* grown strains of *T. denticola* and *P. gingivalis* (9). When this protocol was evaluated clinically, the sensitivity of the PerioScan test relative to clinical disease was 85%

but the specificity was only 40%. The low specificity was due to the detection of the BANA-positive species in the absence of clinical disease (10). We have found the PerioScan test to have a 90–91% sensitivity when it is compared with DNA probes for *T. denticola* plus *P. gingivalis* or with immunological reagents to *T. denticola* plus *P. gingivalis* and *B. forsythus* (11). An unexpected finding was that, with plaque samples, the PerioScan test was comparable to the DNA probes and immunological reagents in the ability to detect low numbers, i.e., 10^4 CFU, of these organisms. From this, we surmised that the BANA-positive species produced more copies of the BANA hydrolytic enzyme per cell *in vivo*. Our protocol, which was based on organisms grown *in vitro*, was too sensitive (in the microbiological sense) in that it was detecting levels of these organisms that were not associated with clinical disease, i.e., carrier state levels.

The detection limit of the PerioScan can be made less sensitive (in the microbiological sense) by shortening the length of incubation and reducing the temperature of incubation. In this investigation, plaques from periodontally healthy individuals were evaluated using the PerioScan test incubated at various times and temperatures. The purpose of this study was to determine the incubation time and temperature that would not detect BANA-positive organisms in plaque samples taken from sites of periodontal health. In this manner, we hoped to establish a protocol that would minimize, if not eliminate, the detection of low or carrier state levels of the BANA-positive species and thereby improve the specificity of the PerioScan test relative to gingival health. In this regard, any positive result would or could be a deviation from health and would cause the clinician to focus attention on those sites.

Material and methods

Subjects and clinical examination

Ninety volunteers were recruited from the students and staff of the School of Dentistry. All volunteers were medically healthy and none had a history of periodontal disease. There were no pockets >4 mm, although some individuals were found to have gingivitis. Gingival health was determined by inserting a wooden toothpick (Stim-udent, Johnson & Johnson Windsor, NJ) into all available interproximal sites mesial to the second molar and recording the onset and magnitude of bleeding according to the papillary bleeding score (PBS) system (5)

PBS 0 = no bleeding, gingival color and contour appear to be healthy;
PBS 1 = no bleeding, gingiva is slightly inflamed and/or slightly red;
PBS 2 = slight bleeding or spotting of blood, no flow along the gingival margin;
PBS 3 = bleeding, blood flow remains confined to the area of the papilla;
PBS 4 = immediate bleeding, blood flow is copious and spreads to the adjacent teeth;
PBS 5 = tissue is grossly inflamed, hemorrhagic and edematous.

Healthy sites had a PBS ≤ 1, i.e., there was no bleeding after insertion of the toothpick, and a diseased site had a PBS ≥ 2, i.e., there was some bleeding after insertion of the toothpick.

BANA test and sampled tooth sites

Interproximal plaque samples were incubated on the PerioScan cards for 5 and 15 min at 35°, 45° and 55°C. Subjects were randomly assigned to the various temperatures. A separate toothpick was inserted interproximally in each of 26 sites, excluding the interproximal site between the 2nd and 3rd molars. Each side of the toothpick was then wiped in a discrete location on a BANA impregnated filter strip that ran along the lower border of the PerioScan card. An upper reagent strip containing Fast Black dye was activated by dampening the strip with water. The lower strip was then folded over the upper strip so that any naphthylamide released from the BANA by plaque enzymes would diffuse into the upper strip where it could react with the Fast Black dye, forming a permanent blue-black color. The reagent card was incubated in a heating block at the above temperatures for 5 min. The card was removed from the heating block and the degree of blue-black color was assessed by eye using the following scale: 1 = negative; 2 = weak positive, a faint blue-black color; 3 = positive, a distinct blue-black color. Only the positive reactions were recorded, as we wanted to determine whether the weak-positive reactions became positive upon further incubation. The cards were replaced in their respective heating blocks and incubated for an additional 10 min and then the color was again recorded to give a 15-min value. A weak-positive and a positive recording at the 15-min incubation period were both considered as positive for purposes of statistical analysis.

Statistics

For a true-positive result, the PerioScan reagent card had to be positive in the presence of a PBS ≥ 2; for a true-negative result, the PerioScan reagent card had to be negative in the presence of a PBS ≤ 1. Plaques that were reagent card-negative and PBS positive were considered false negatives, whereas plaques that were reagent card-positive and PBS negative were scored as false positives. The accuracy of the PerioScan reagent card test was determined by the sum of the true-negative results plus the true-positive results as divided by the total numbers of plaques sampled. The odds ratio was obtained by multiplying the true-positive (TP) times the true-negative (TN) and dividing that result

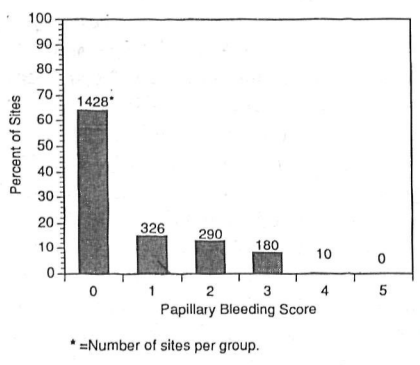

Fig. 1. Distribution of sites per papillary bleeding score. There were 25 sites per patient.

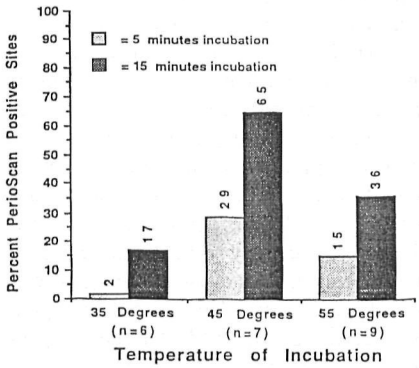

Fig. 2. Distribution of PerioScan positive sites as a function of incubation time and temperature in subjects with no bleeding sites. *n* is number of patients. There were 25 sites sampled per patient.

Table 1. Characteristics of patient population

	Number of subjects	Mean age (years)	Age range	Women	Men
35°C	33	30.5	21–51	11	22
45°C	28	30.1	22–47	13	15
55°C	29	30.6	16–55	10	19
Totals	90	30.4		34	56

by the product obtained by multiplying the false-positives (FP) and the false-negative (FN) (TP × TN/FP × FN). Receiver-operating characteristic (ROC) curves were obtained by plotting the specificity (TN) of the test for various times and temperatures of incubation against the FN values (4).

Results

There were no age or gender differences among the 90 subjects assigned to the 3 temperature groups (Table 1). The subjects averaged about 30 years of age and men outnumbered women. Seventy-nine percent of the 2234 measured interproximal sites were periodontally healthy, i.e., PBS ≤ 1 (Fig. 1). Only 9% of the sites exhibited an obvious gingivitis characterized by bleeding with a flow, i.e., PBS ≥ 3.

There were 23 subjects who exhibited no gingival bleeding, and who were disease-free, i.e., no pockets ≥3 mm, no attachment loss. The distribution of the percentage of PerioScan-positive sites among these disease-free subjects is shown in Fig. 2. Only 2% of the plaques were PerioScan-positive when the cards were incubated for 5 min at 35°C, compared with 29% and 15% respectively when the cards were incubated at 45°C and 55°C. The percentage of PerioScan-positive plaques at all temperatures increased at 15 min, with 65% of the plaques being positive when incubated for 15 min at 45°C (Fig. 2).

The relationship between PBS and a positive PerioScan score as a function of the temperature and length of incubation for all the subjects is shown in Fig. 3. When the gingival tissue was healthy, i.e., PBS = 0, only 3% of the plaques were BANA-positive after 5 min of incubation, and 6% were positive after 15 min of incubation at 35°C (Fig. 3). However, at 45°C and 55°C, from 25 to 60% of the plaques were BANA-positive. When plaques removed from the PBS = 1 sites were similarly analyzed, only 6% of the plaques were positive when the PerioScan cards were incubated for 5 min at 35°C (Fig. 3). At the higher temperatures and/or longer incubation period, 35–78% of the plaques were BANA-positive. The higher number of PerioScan-positive plaques at 45° and 55°C could be attributed to the increased number of weakly BANA-positive plaques observed, i.e., 3–5 times more than observed at 35°C. A similar pattern was observed when the plaques were taken from a PBS = 2 site. When obvious gingival inflammation was present, i.e., PBS = 3, 30–55% of the plaques were BANA-positive when incubated at 35°C for 5 min, but 95% of the plaques were BANA-positive after 15 min of incubation at 55°C.

The sensitivity and specificity of the PerioScan test relative to gingival health at the various incubation temperatures and times are shown in Table 2. We defined health as the absence of any bleeding upon the interproximal insertion of the toothpick, i.e., PBS ≤ 1, and gingivitis as the presence of any bleeding, including spotting, i.e., PBS ≥ 2. The best sensitivity value was obtained when the cards were incubated for 15 min at 55°C, i.e., 85%, but the specificity under these conditions was 50%. When the PerioScan cards were incubated for only 5 min at 35°C, the specificity was 96% but the sensitivity was low (Table 2).

The PerioScan test was more accurate, i.e., able to correctly identify true-positives plus true-negatives when incubated at 5 min instead of 15 min, with the best accuracy value occurring when incubated for 5 min at 35°C (Table 3). The odds of obtaining a correct reading relative to health or disease were 6.7 when the PerioScan card was incubated for 5 min at 35°C, and 5.8 when incubated for 15 min at 55°C (Table 3). Other analyses showed that a ROC curve of the data in Table 2 demonstrated an inflection point after 5 min of incubation at 55°C, suggesting that that this time and temperature might be the best compromise for specificity and sensitivity (Fig. 4).

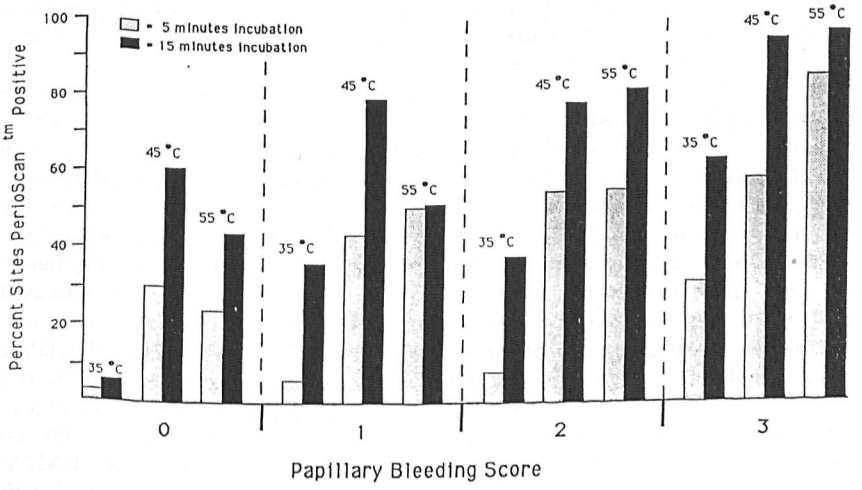

Fig. 3. Effect of gingival health and the length and temperature of incubation on the PerioScan scores. The PerioScan cards were incubated for 5 and 15 min at either 35°C, 45°C or 55°C. No papillary bleeding scores = 4 plaques were incubated at 45°C or 55°C.

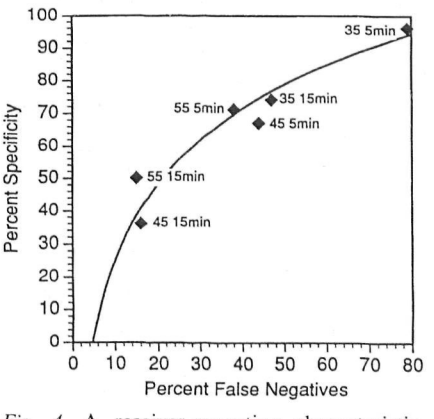

Fig. 4. A receiver-operating characteristic (ROC) curve resulting from the plotting specificity at various time and temperatures against the false-negative ratio. The values are taken from Table 2.

Table 2. Sensitivity and specificity of PerioScan test at various temperatures and length of incubation

	5 min		15 min	
PerioScan	PBS≤1	PBS≥2	PBS<1	PBS≥2
		35°C		
Negative	586	168	445	101
Positive	24	46	165	113
Sensitivity	21%		53%	
Specificity	96%		74%	
		45°C		
Negative	387	52	207	19
Positive	191	65	371	98
Sensitivity	56%		84%	
Specificity	67%		36%	
		55°C		
Negative	403	57	284	22
Positive	163	92	282	127
Sensitivity	62%		85%	
Specificity	71%		50%	

Discussion

The actual levels at which an organism can be associated with or cause periodontal disease are not known, but have been estimated to be about 10^6 organisms in any given pocket (9). The optimum time and temperature of incubation to detect about 10^6 CFU of *P. gingivalis* and *T. denticola* was 15 min at 55°C when laboratory grown strains were used (9). This finding led to the choice of these conditions for the clinical evaluation of the PerioScan test. However, the appearance of false-positive Perio Scan results, i.e., low specificity, in plaque samples removed from periodontally healthy sites (10), suggested that *in vivo* lower levels of the BANA-positive species were actually being detected. These levels of *P. gingivalis, B. forsythus* and *T. denticola* were subsequently found to be about 10^4 CFU when highly specific DNA probes and antibodies to these organisms were used (11). Apparently, the 15-min incubation period at 55°C provided these low numbers of *in vivo* growing BANA-positive organisms with sufficient time to hydrolyze the BANA substrate so as to yield a detectable color reaction. This meant that a new protocol had to be developed that would give a negative PerioScan result in the presence of clinical health and a positive PerioScan result in the presence of disease.

Table 3. The odds ratio and accuracy of the PerioScan test at different incubation times and temperatures

	Time of incubation	
Temperature of incubation	5 min	15 min
	Odds ratio (TP×TN/FP×FN)	
35°C	6.7	3.0
45°C	2.5	2.9
55°C	4.0	5.8
	Accuracy (TP+TN/Total)	
35°C	77%	68%
45°C	65	44
55°C	69	57

We approached this problem in the present investigation by determining the length and temperature of incubation that would give negative PerioScan results in young people with no prior history of periodontal disease. We reasoned that the incubation conditions that would give negative PerioScan results in a clinically healthy population would provide guidance for, if not define, the conditions for obtaining the maximal specificity of the PerioScan test. Of the 90 people studied, there was a subgroup of 23 individuals who were disease-free. Only the 5-min incubation at 35°C protocol gave essentially negative PerioScan reactions in these individuals (Fig. 2). Incubations at higher temperatures and for longer periods increased the number of PerioScan-positive results (due mostly to the presence of weak-positive results) to as high as 65% at the 15 min, 45°C protocol. This indicated how crucial the incubation conditions were for the non-detection of the BANA-positive species in plaque samples.

The above result indicated that a 5-min incubation at 35°C would identify periodontally healthy sites as being BANA-negative. This finding was evaluated in the larger population of 90 subjects who participated in this investigation. The findings in these individuals indicated that, under these conditions, 96% of the clinically healthy sites were PerioScan-negative, and contrasted markedly with a specificity of 50% that was observed when the PerioScan cards were incubated for 15 min at 55°C (Table 2). However, with the 5 min and 35°C incubation format there is reduced sensitivity, especially compared with the 85% sensitivity observed at the 15 min and 55°C incubation format. Thus, under conditions that essentially eliminated any false-positive reactions, an untoward number of false-negative reactions were encountered.

In order to minimize both the false-positives and the false-negatives, a compromise time and temperature might be indicated, such as the 5 min-55°C degree format that seems best when an ROC curve was calculated (Fig. 3). However, the sensitivity of 62% obtained under these conditions is not good. This suggests that a dual incubation schedule may be necessary, i.e., incubate for 5 min at 35°C followed by another 10 min at 55°C. If the plaque is negative at both temperatures, then most likely the BANA-positive organisms collectively are $<10^4$ CFU and there may be minimal risk of infection due to these organisms. If the plaque is negative at 35°C, but positive at 55°C, then the organisms are present in carrier state levels, i.e., $>10^4$, but $<10^6$ CFU. The clinician would use this information to better advise the patient concerning oral hygiene procedures. If the plaque is BANA-positive at 5 min, then that tooth site is colonized by levels of the BANA-positive species that are usually associated with periodontal disease, i.e., $>10^6$ CFU. The clinician would use this information in developing the treatment plan.

In previous studies in which the PerioScan exhibited a high sensitivity, the source material was subgingival plaque removed mainly from diseased sites with probing depths >4 mm, and the sensitivity was determined relative to the presence of periodontitis (9, 10). In the present study, the high sensitivity was made relative to gingivitis and raises the issue as to whether the BANA-positive organisms are associated with gingivitis as well as periodontitis. Although it is generally accepted that most

sites with gingivitis do not proceed to periodontitis and that there can be periodontitis without concurrent gingivitis, these observations do not preclude the possibility that most, if not all, forms of periodontitis are preceded by a form of gingivitis. (The exception to this might be localized juvenile periodontitis.) The present findings that BANA-positive organisms found in interproximal plaque samples can be associated with gingivitis emphasizes the need to treat gingivitis to prevent the possible subsequent appearance of periodontitis.

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