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Development of a Diagnostic Test for Anaerobic Periodontal Infections Based on Plaque Hydrolysis of Benzoyl-DL-Arginine-Naphthylamide

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***Treponema denticola, Porphyromonas (Bacteroides) gingivalis*, and *Bacteroides forsythus* are among the anaerobic species frequently associated with adult forms of periodontal disease. These organisms hydrolyze the synthetic peptide benzoyl-DL-arginine-naphthylamide (BANA), and such enzyme activity can be detected in the plaque and related to clinical disease and the presence of spirochetes. In this investigation, the liquid BANA assay was compared with a commercially developed BANA assay which employed a paper format and which could be read after a 15-min incubation. In the paper format, strips of a Whatman filter paper were impregnated with BANA and strips of nitrocellulose paper were impregnated with fast black K salt. Both strips were applied lengthwise across a paper card (3 by 5 in. [7.6 by 12.7 cm]). The BANA strip at the bottom was inoculated with the test sample (pure culture, plaque), folded back so that it contacted the fast black strip, and then incubated for 15 min at 55°C. *T. denticola*, *P. gingivalis*, and *B. forsythus* always gave a positive reaction, whereas 51 other plaque species were always negative. Six *Bacteroides* and *Capnocytophaga* species on occasion had weak reactions. The proportional agreement between BANA positiveness and clinical disease was similar for both the liquid and the paper assays. The sensitivity, specificity, and accuracy relative to the clinical standard of the liquid assay were 74, 76, and 77%, respectively, while those of the paper assay were 81, 78, and 80%, respectively. The paper assay was significantly associated with the presence of either *T. denticola* or *P. gingivalis* or both in the plaque samples, with a sensitivity of 85%, a specificity of 53%, and an accuracy of 79%. These findings indicate that a rapid paper assay for BANA hydrolysis gives data comparable to those obtained with the liquid BANA assay.**

Treponema denticola, Porphyromonas (Bacteroides) gingivalis (17), and *Bacteroides forsythus* are among the bacterial species frequently associated with adult forms of periodontal disease (3, 9, 13). These organisms are gram-negative asaccharolytic anaerobic species that appear to be unique among the bacteria which reside on the tooth surfaces in that they possess an enzyme which hydrolyzes the synthetic peptide benzoyl-DL-arginine-naphthylamide (BANA) (8). This enzyme activity is detectable in subgingival plaque samples and has been statistically associated with the levels and proportions of spirochetes in the plaque (1, 12), with pocket probing depth (6, 12), with clinical disease at the sampled site (16, 20; W. A. Bretz, D. E. Lopatin, and W. J. Loesche, Oral Microbiol. Immunol., in press), and with the immunological detection of *T. denticola* and *P. gingivalis* in the same plaque samples (Bretz et al., in press). The BANA substrate is the most accurate of several synthetic naphthylamide substrates in reflecting clinical disease in untreated periodontal patients (20). Moreover, the enzymatic activity for equal amounts of plaque was higher in diseased patients than in clinically treated and maintained patients (6).

In these studies (12, 16; Bretz et al., in press), plaque samples were collected from single sites, dispersed, and diluted and suitable portions of the plaque suspension were incubated overnight with the BANA substrate. The color was developed by the addition of fast garnet (7). This simple procedure does not lend itself readily to a clinical setting, as the reagents must be freshly prepared from stock solutions and the 18- to 24-h incubation does not permit an immediate application of the test results to the management of the patient. In this study, a commercially developed BANA assay, which employs a solid-state format and can be read after a 15-min incubation period, was compared with the original BANA assay.

MATERIALS AND METHODS

Pure cultures. Pure cultures of *P. gingivalis* ATCC 33277 and recent isolates, strains W and T, were grown for 4 days in enriched Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) (11). *T. denticola* ATCC 35405 and recent isolate ASLM were grown in a tryptone-veal heart infusion-yeast extract medium (14) for 5 to 7 days. The cells were harvested by centrifugation, washed once in reduced transport fluid (10), and resuspended in reduced transport fluid to give a final suspension of about 10^9 CFU/ml. In experiments determining the detection limits of the BANA assay, the cell suspensions were diluted 1:5, 1:10, 1:50, 1:100, 1:500, 1:1,000, and 1:5,000 in reduced transport fluid. Samples of 5 μl of the various dilutions were applied directly to the BANA strip contained on the Perioscan reagent card (see below).

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The following plaque species were American Type Culture Collection (Rockville, Md.) and Forsyth Dental Center reference strains as well as fresh isolates. These species were Perioscan positive (number of strains tested): *Treponema denticola* (3), *Porphyromonas* (*Bacteroides*) *gingivalis* (16), and *Bacteroides forsythus* (11). These species were variable in the Perioscan test: *Bacteroides capillosus* (2), *Bacteroides denticola* (1), *Bacteroides oralis* (6), *Capnocytophaga gingivalis* (7), *Capnocytophaga ochracea* (9), and *Capnocytophaga sputigena* (3). These species were Perioscan negative: *Actinobacillus actinomycetemcomitans* (12), *Actinomyces israelii* (7), *Actinomyces naeslundii* (12), *Actinomyces viscosus* (6), *Bacteroides asaccharolyticus* (1), *Bacteroides buccae* (1), *Bacteroides denticola* (1), *Bacteroides gracilis* (4), *Bacteroides heparinolyticus* (1), *Bacteroides intermedius* I (7), *Bacteroides intermedius* II (6), *Bacteroides loescheii* (1), *Bacteroides melaninogenicus* (5), *Bacteroides oris* (1), *Bacteroides ureolyticus* (1), *Bacteroides oulorum* (1), *Bacteroides zoogleoformans* (1), *Campylobacter concisus* (4), *Campylobacter sputorum* (1), *Eikenella corrodens* (13), *Escherichia coli* (2), *Fusobacterium nucleatum* subsp. *nucleatum* (11), *Fusobacterium nucleatum* subsp. *polymorphum* (7), *Fusobacterium nucleatum* subsp. *vincenti* (5), *Fusobacterium periodonticum* (1), *Haemophilus aphrophilus* (9), *Haemophilus paraphrophilus* (2), *Haemophilus segnis* (1), *Lactobacillus acidophilus* (1), *Peptostreptococcus micros* (7), *Propionibacterium acnes* (5), *Selenomonas artemidis* (1), *Selenomonas flueggeii* (1), *Selenomonas noxia* (1), and *Selenomonas sputigena* (1), *Streptococcus constellatus* (8), *Streptococcus intermedius* (11), *Streptococcus mitis* (8), *Streptococcus morbillorum* (11), *Streptococcus mutans* (7), *Streptococcus salivarius* (10), *Streptococcus sanguis* I (13), *Streptococcus sanguis* II (7), *Streptococcus sobrinus* (4), *Streptococcus uberis* (5), *Treponema pectinovorum* (1), *Treponema vincentii* (1), *Veillonella parvula* (6), *Wolinella cura* (2), *Wolinella recta* (8), *Wolinella succinogenes* (1). Reference strains were revived from storage in liquid nitrogen, checked for purity, and confirmed as the designated species by using phenotypic characteristics (2, 3). Fresh isolates were obtained from subgingival plaque samples, characterized, and identified as described previously (2, 3). When multiple strains of a species were tested, every isolate was obtained from a different subject. A total of 295 strains representing 57 species were tested, as summarized below.

The organisms were grown on Trypticase soy agar plates supplemented with 5% sheep blood and incubated at 35°C in an atmosphere of 80% N_2, 10% H_2, and 10% CO_2 for 2, 5, or 10 days. A large loopful of growth was removed from the surface of the agar plates with a platinum loop and placed on the Perioscan test strips. The strips were incubated at 55°C. All isolates were tested in duplicate on three separate occasions, i.e., after 2, 5, or 10 days of incubation. The reaction was scored as strongly positive when a large area of blue approximated the deposited cell mass. Weak or questionably positive was recorded when reactions were small areas of blue at localized spots approximating the bacterial deposit. When discrepancies occurred between replicate experiments of the same strain or between strains of the same species, the purity of each strain was reconfirmed and the BANA reaction was reassessed.

Plaque samples. Either supragingival or subgingival plaque samples were collected with a periodontal curette from single tooth sites in patients diagnosed as having advanced periodontitis and placed immediately into a plastic vial containing 0.1 to 0.5 ml of reduced transport fluid. The probing depth of each sample site was measured with a thin metal rod that was calibrated in millimeters (a periodontal probe), and it was noted whether the site bled upon probing. The clinician was then required to state whether the site was healthy or diseased, basing this judgement on the probing depth, bleeding tendencies, and tissue appearance (16). A diseased site was one which exhibited bleeding on probing and had a probing depth of ≥4 mm. A healthy site was one in which there was no bleeding upon probing.

Microscopic counts. The plaque samples were dispersed for 20 s with a vortex mixer, and 10 μl was removed for microscopic examination. Either 200 organisms or the number of organisms in 20 high-power fields (hpf) were enumerated, depending on which event occurred first. The single cells were identified as spirochetes, selenomonads, vibrio-like motile rods, fusiforms, nonmotile rods, or cocci (11).

BANA analysis. The BANA analysis was performed in three formats: the standard liquid assay, a solid-state sandwich assay, and a solid-state card assay.

The protocol for the standard assay has been described previously (1, 12) and will be briefly summarized. A stock solution of BANA (44 mg in 1 ml of dimethyl sulfoxide) was diluted 1 to 100 in Sorensen buffer to give a working solution of 0.67 mM BANA. Portions of 100 μl of this BANA solution were added to 100 μl of the various bacterial or plaque suspensions contained in a well of a plastic plate (Minitek; BBL Microbiology Systems). The mixture was incubated overnight, and then the color was developed by the addition of 50 μl of a fast garnet solution. The resulting yellow-to-red color was read in early studies by eye as negative (yellow), weakly positive (yellow-orange), or positive (orange to red). In subsequent studies, the color was read with an enzyme-linked immunosorbent assay (ELISA) reader with a 405-nm filter, and an absorbance of less than 0.10 was recorded as a negative result.

A prototype sandwich technique was developed in collaboration with Oral-B Laboratories, Inc. (Redwood City, Calif.) in which Whatman filter paper was impregnated with BANA and nitrocellulose paper was impregnated with fast black K salt. Fast black was substituted for the fast garnet because of its superior stability once the color was developed and because its blue-black color, unlike the red-orange color of fast garnet, is easily distinguishable from the color of blood. Preliminary experiments with *P. gingivalis* and *T. denticola* showed that the maximal color development occurred when a 1:50 dilution of the above-described stock BANA solution in 15 mM Tris hydrochloride (pH 8.5) was added to the filter paper (paper 903; Schleicher & Schuell, Inc., Keene, N.H.) and a 0.2% fast black K salt was added to the nitrocellulose paper (pure nitrocellulose BA 95; Schleicher & Schuell).

In the sandwich assay, it was necessary to concentrate the plaque suspension. Accordingly, from 50 to 100 μl was removed and centrifuged for 5 min (microCentrifuge model 235C; Fisher Scientific Co., Pittsburgh, Pa.). The supernatant fluid was discarded, and the pellet was suspended in 5 μl of phosphate-buffered saline. This suspension was spotted on the BANA-treated paper. After the BANA paper had been inoculated, the fast-black-treated nitrocellulose paper was moistened with water and placed over the BANA paper, and the papers were clamped together with paper clips. These sandwich preparations were incubated for various times and temperatures. In the described experiments, the preparations were incubated for 30 min at 45°C, unless stated otherwise. A faint blue or blue-black color in the fast black paper was considered a weak positive reaction, and a

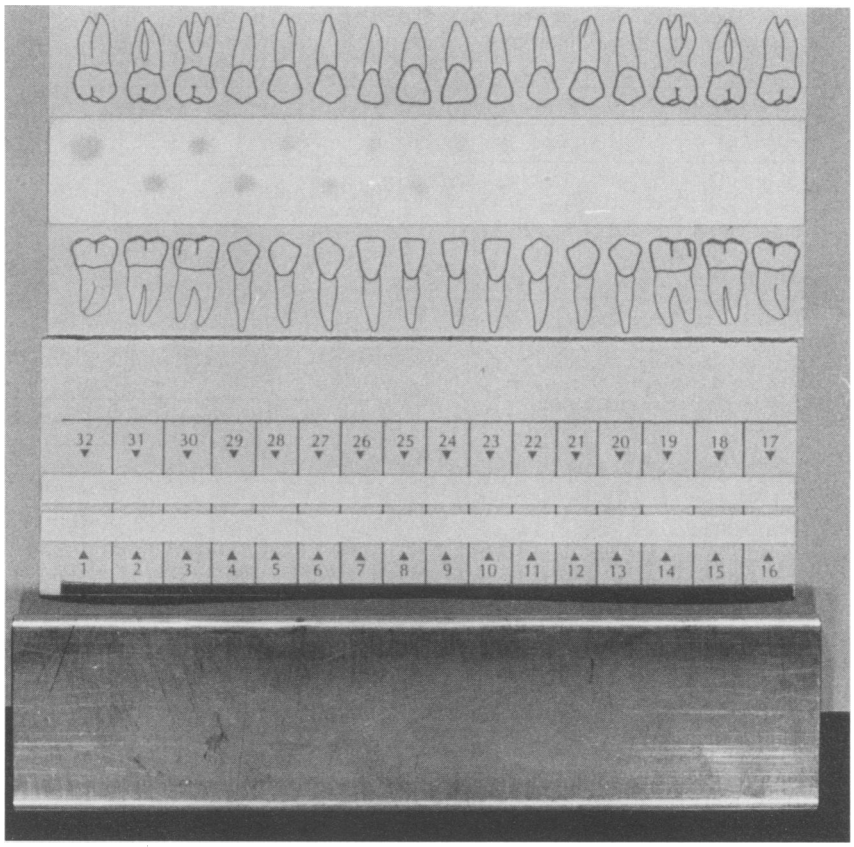

FIG. 1. Perioscan card showing positive BANA reactions at different bacterial concentrations of *P. gingivalis* ATCC 33277. Serial dilutions of the strain were alternately placed on either the upper or the lower BANA-impregnated strip, beginning at sites numbered 1, 31, 3, 29, etc. The strip containing fast black, which is positioned between the drawings of the teeth, was moistened with water, and then the BANA strips were folded up on the fast black strip. The folded card was placed in the metallic clip shown at the bottom and then incubated in a heat block at 55°C for 15 min. The positive reactions were observed on the fast black strip, with the strongest reaction at site 1.

distinct blue or blue-black color was considered positive. This assay was able to detect 5 to 10 ng of trypsin per μl (bovine pancreatic trypsin type III; specific activity, approximately 11,000 BAEE units/mg of protein; Sigma Chemical Co., St. Louis, Mo.).

The card assay was an extension of the sandwich technique and represented a commercial product (Perioscan) that was developed in conjunction with Oral-B Laboratories. The BANA- and fast-black-impregnated papers were applied as strips that ran lengthwise across a card (3 by 5 in. [7.6 by 12.7 cm]) (Fig. 1). The BANA strips at the bottom could be folded up so that they contacted the fast black strip. This contact was maintained by a metallic clip into which the folded card could be inserted. This card format was studied to determine the sensitivity of the assay for various pure cultures. The pure cultures were concentrated by centrifugation, and 5 μl of each culture was slowly applied as a discrete spot on the BANA strip. The fast black strip was moistened with distilled water, and then the BANA strip was folded up onto it and held in place by the clip (Fig. 1). In the described experiments, the BANA card was incubated for 15 min at 55°C unless stated otherwise. The cards were read as follows: negative, absence of a blue or blue-black color; weakly positive, a faint blue or blue-black color; positive, a discrete blue-black color.

Various pure cultures of *T. denticola* and *P. gingivalis*, plaque suspensions, and a trypsin standard were inoculated in this fashion and incubated for various times and temperatures. Pure cultures were grown to the early stationary phase, concentrated by centrifugation, suspended in phosphate-buffered saline, and diluted in phosphate-buffered saline. A 5-μl sample of each of three dilutions per organism was spotted along with a trypsin standard solution onto a single card. Enough cards were inoculated to incubate duplicate cards for 5, 15, 30, and 60 min at 25, 35, 45, 55, 65, or 75°C. The dilutions chosen included a cell count known to yield a positive reaction in the sandwich technique, a cell count that was 8- to 10-fold higher than this positive control, and a cell count that was 500-fold lower, which served as a negative control. The trypsin standard was tested at 10 and 1.3 ng/μl.

ELISA. The lower reagent strip containing the plaque was removed from the Perioscan card, and then the strip was cut in half and each half was incubated in a solution of highly specific hyperimmune rabbit antibodies to either *P. gingivalis* or *T. denticola*. The strips were then washed in phosphate-buffered saline-Tween buffer and then incubated in alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. The immune complexes were revealed after incubation with BCIP/NBT phosphatase substrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). This ELISA procedure will detect about 5×10^4 CFU of either *T. denticola* or *P. gingivalis*, which is about 10-fold more sensitive than the Perioscan. The specificity of the antibod-

TABLE 1. Relationship between BANA hydrolysis as detected by fast garnet (liquid) and fast black (paper) and the levels of spirochetes in plaque samples

Test result	No. of spirochetes/hpf[a] with:	
	Fast garnet	Fast black
Negative	1.6 ± 3.5 (43)	1.8 ± 3.6[b] (44)
Weakly positive	14.1 ± 5.4 (18)	8.8 ± 10.8 (5)
Positive	30.6 ± 3.7[c] (38)	27.2 ± 3.4[b] (50)
ANOVA[d]	$P = 0.0002$	$P = 0.0145$

[a] Average ± standard error. The number of plaque samples is shown in parentheses.
[b] Values are significantly different from each other.
[c] Value significantly different from other values in the same column.
[d] Two-factor ANOVA with one random factor being the patient.

TABLE 2. Comparison of liquid and paper assays for BANA hydrolysis in subgingival plaques removed from periodontally healthy and diseased sites

Assay and site	% Positive plaques	% Negative plaques	Total plaques (%)	% Agreement[a]
Liquid (fast garnet)				77
Diseased	74	26	46 (100)	
Healthy	21	79	14 (100)	
Total no. of plaques	38	22	60	
Paper (fast black)				80
Diseased	80	20	46 (100)	
Healthy	14	86	14 (100)	
Total no. of plaques	39	21	60	

[a] Observed proportional agreement estimates were calculated by the correlated binomial model (Hujoel et al., in press).

ies and the details of the protocol are described elsewhere (Bretz et al., in press). The ELISA reactions were graded as negative, weakly positive, or positive.

Statistics. Each of the data sets, i.e., enzymatic, clinical, and microscopic, was determined independently and then compiled for statistical analysis. A two-factor analysis of variance (ANOVA) (P. Hujoel, L. Moulton, and W. Loesche, J. Periodontal Res., in press) was performed on data obtained with the Perioscan, with the liquid BANA assay, and with the sandwich format assay. In this analysis, the independent factor was the BANA assay or ELISA (negative, weakly positive, or positive) (fixed factor), the patient was a random factor, and the dependent variables were the various plaque parameters under investigation. The sensitivity, specificity, and accuracy of the Perioscan test results were determined relative to those of the ELISA as a bacterial standard for *T. denticola* and *P. gingivalis*. All these estimates were calculated by the correlated binomial model, which takes into account the fact that sites within a patient are correlated and allows for a heterogeneous number of responses within a patient (Hujoel et al., in press). Similarly, the observed proportional agreement of the Perioscan, the liquid BANA assay, and the sandwich assay with the clinical status of the sampled site and the observed proportional agreement between the Perioscan and the liquid BANA assay were calculated by the correlated binomial model.

RESULTS

Preliminary experiments had shown that a procedure in which inocula of *P. gingivalis* and *T. denticola* were sandwiched between a filter paper impregnated with BANA and a nitrocellulose paper impregnated with fast black gave rise to a blue or blue-black color in the nitrocellulose paper. The color was permanent and could be developed within 15 to 60 min, depending on the incubation temperature (between 37 and 60°C). A temperature of 45°C and a 30-min incubation period were chosen for an experiment in which supragingival and subgingival plaque samples were analyzed by both the liquid assay with fast garnet as the color developer and the paper sandwich assay with fast black as the developer (Table 1). Both the liquid and sandwich assays yielded comparable information relative to the levels of spirochetes that were present in a microscopic hpf (one organism per hpf is equivalent to approximately 5×10^6 cells per ml). A positive reaction was associated with about 27 to 30 spirochetes per hpf, while a negative reaction was associated with fewer than 2 spirochetes per hpf. Of the 56 positive plaques with the fast garnet developer, 18 were judged as weakly positive, whereas only 5 of the 55 positive plaques in the fast black reaction were judged as weakly positive. As the intensity of color was determined subjectively by visual examination, this suggested that the viewer was better able to recognize positive reactions with the fast black developer in the sandwich assay.

The liquid assay has been shown to agree significantly with the assessment by the clinician of health or disease in the sampled tooth site (16; Bretz et al., in press). In the present experiment, the proportional agreement between BANA positiveness and disease was similar for both the liquid and the paper assays (Table 2). The sensitivity (true positive), specificity (true negative), and accuracy [(true positive + true negative)/total number] relative to the clinical standard (disease being positive, health being negative) of the liquid assay were 74, 76, and 77%, respectively, while those of the paper assay were 81, 78, and 80%, respectively. These percentages were slightly lower than the values shown in Table 2, which were calculated directly from the number of plaques examined without taking into account that some of the plaques were clustered within patients (Hujoel et al., in press).

Thus, the paper assay was essentially identical to the liquid assay. Since the paper assay could be performed in 30 min, these results suggested that it would be possible to obtain a BANA test result during the same clinical visit in which the plaque was collected. The paper assay was further modified to facilitate its clinical use. The awkward sandwich procedure was replaced by the Perioscan card in which the plaque could be placed on the BANA strip and then folded up to make contact with the fast black strip. The drawings of the teeth were added to facilitate interpretation of the results (Fig. 1).

The optimal temperature of incubation and length of incubation were determined with American Type Culture Collection strains of *T. denticola* and *P. gingivalis*. The heavy cell suspension containing 62×10^6 CFU of *T. denticola* per 5 μl reached maximum color intensity after 30 min of incubation at 35°C but needed only 5 min of incubation at 55°C (Fig. 2). The cell suspension containing 5×10^6 CFU/5 μl reached its maximum color intensity after 15 min of incubation at 55°C. This level, which approximated the level of *T. denticola* seen in most plaque samples from a diseased tooth site, exhibited a temperature optimum at 55°C. The light cell suspension of *T. denticola*, i.e., 1.5×10^4 CFU/5 μl, was negative at all times and at all temperatures. The BANA enzyme in *P. gingivalis* appeared to be more

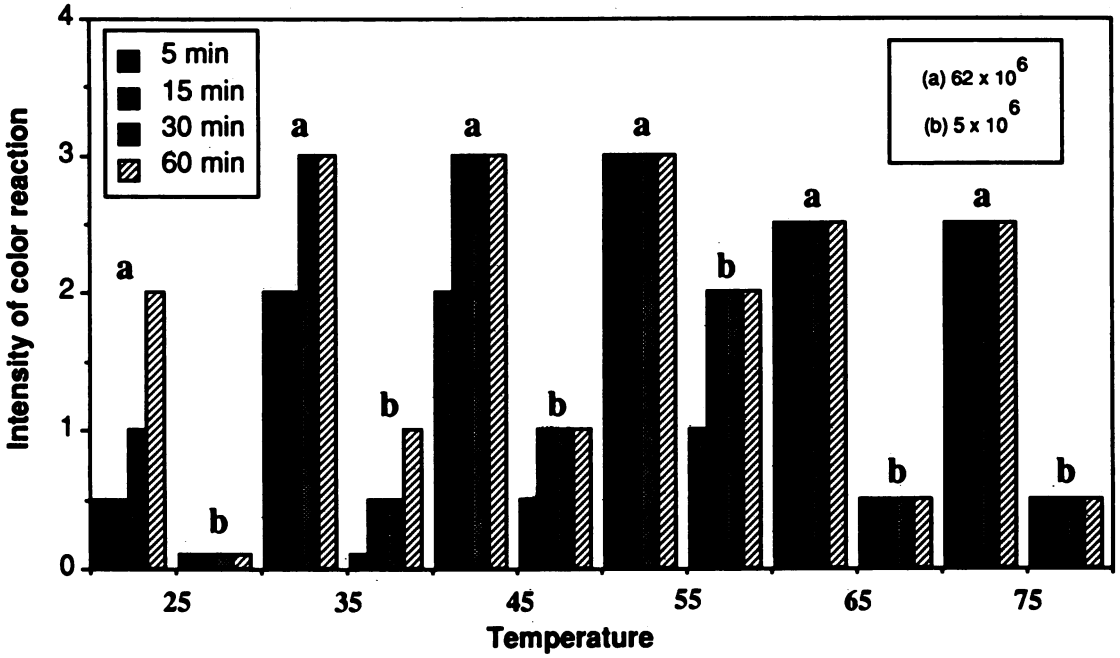

FIG. 2. Bar graph showing intensity of BANA color reaction as a function of incubation temperature (°C), time of incubation, and number of cells of *T. denticola* ATCC 35405.

heat stable than the *T. denticola* enzyme, as the maximum color intensity with the heavy cell suspension occurred within 5 min at 65°C (Fig. 3). At levels of 9×10^6 CFU/5 μl, the color was less intense and did not increase with either the length or temperature of incubation beyond that seen after 15 min of incubation at 45°C. Trypsin (10 ng/μl) was positive at all temperatures after 15 min of incubation (data not shown).

These findings led to a selection of 15 min at 55°C as the standard protocol in which we evaluated the detection limits of the Perioscan card for two strains of *T. denticola* and three strains of *P. gingivalis*. The cell count that was one fivefold dilution below the last weakly positive BANA reaction was selected as the detection limit. Variation was noted between different strains of the same species, as well as within different replications of the same strain (Table 3). On the average, about 1×10^6 CFU of *T. denticola* were required to give a weakly positive reaction, whereas about 2×10^5 CFU of *P. gingivalis* were required.

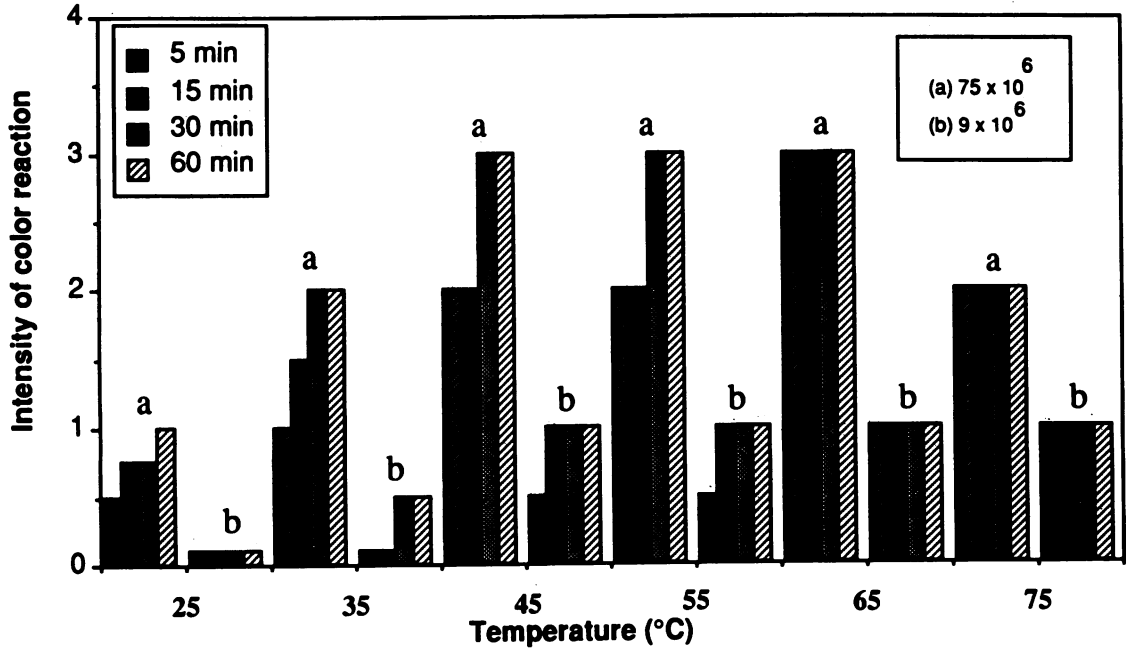

FIG. 3. Bar graph showing intensity of BANA color reaction as a function of incubation temperature, time of incubation, and number of cells of *P. gingivalis* ATCC 33277.

TABLE 3. Detection limits for Perioscan test for *T. denticola* and *P. gingivalis*

Strain	10^5 CFU[a]	Range (10^5)	No. of replications
T. denticola			
ATCC 35405	8.4 ± 12.0	0.2–57.5	21
ASLM	21.8 ± 15.4	6.6–49.6	8
P. gingivalis			
ATCC 33277	3.3 ± 2.9	0.8–0.0	12
T	1.5 ± 1.3	0.3–5.2	12
W	1.2 ± 0.8	0.05–2.7	12

[a] Mean ± standard deviation.

TABLE 5. Agreement between Perioscan BANA results and liquid BANA results[a]

Liquid BANA result	% Perioscan positive[b]	% Perioscan negative[b]	Total plaques
Positive	88	18	57
Negative	12	82	127
Total plaques (%)	34 (100)	150 (100)	184

[a] Observed proportional agreement of 83% was calculated by the correlated binomial model (Hujoel et al., in press). The kappa value was 0.58.
[b] Proportion of plaques.

A large panel of reference strains was then evaluated for its ability to give a BANA reaction in the Perioscan format. Three patterns were observed. Only strains of *P. gingivalis*, *B. forsythus*, and *T. denticola* exhibited consistent positive reactions (see Materials and Methods). Thirty strains of these species, tested on at least three occasions, uniformly yielded positive reactions. Most species gave a BANA-negative reaction. Thus, 244 strains representing 51 species did not exhibit any BANA-positive reactions. Two spirochetes, *T. vincentii* and *T. pectinovorum*, as well as four black-pigmented bacteroides species, i.e., *B. intermedius* I, *B. intermedius* II, *B. loescheii*, and *B. melaninogenicus*, were uniformly BANA negative.

A variable reaction was observed with certain *Bacteroides* and *Capnocytophaga* species in which 36 of 152 separate determinations were positive or weakly positive (see Materials and Methods). The inconsistent reactions of this group of strains led to the following experiments. Initially, all cultures were confirmed for purity on the basis of colonial and microscopic examination and biochemical tests. Thus, inconsistency could not be attributed to mixed cultures or misidentification. The relationship of time and incubation of the test strains on blood agar plates with the subsequent positive or negative test reaction was examined, but no clear relationship could be established. Cells grown in broth and harvested by centrifugation were no more consistent in their reaction than were cells harvested from agar surfaces. Even the use of extremely large cell masses (as much as could be physically placed on the strip) did not result in consistent positive reactions for these test strains.

In subsequent experiments, subgingival plaques were removed from periodontally involved teeth and, after dispersion, equal portions were evaluated by the liquid BANA and Perioscan assays. The majority of the plaques were Perioscan negative, and this coincided with the inability of the plaque to hydrolyze BANA in the liquid assay and with the presence of low levels and proportions of spirochetes in the plaque samples (Table 4). When the data were analyzed by a two-factor ANOVA, there were highly significant differences among the Perioscan scores and (i) BANA hydrolysis, (ii) total spirochetes in the sample, (iii) spirochetes per hpf, and (iv) percent spirochetes (Table 4). The clearest differences were observed with the BANA hydrolysis and with the total spirochetes in the sample, as their value at each Perioscan score was significantly different from their values at the other Perioscan scores. This was to be expected if the Perioscan and liquid BANA assay were measuring the same enzyme activities and if most of this activity was due to the spirochetal, i.e., *T. denticola*, load of the sample (12).

The liquid BANA and the Perioscan BANA results were related to each other with an 83% agreement (Table 5). The main difference between the tests was the greater number of positive reactions found in the liquid assay, i.e., 57 versus 34 in the Perioscan assay. This could be attributed to the higher number of weakly positive results detected in the liquid assay, i.e., 31 versus 16 for the Perioscan assay. This suggested that the overnight incubation period for the liquid BANA was permitting the detection of small numbers of BANA-positive organisms that apparently could not be detected during the 15-min incubation used in the Perioscan assay.

The inoculum used for both BANA assays in these experiments was only a fraction of the plaque suspension and not the entire plaque sample. In practice, however, the entire plaque sample would be used for the Perioscan assay. We next evaluated the relationship between the Perioscan results and clinical status when the entire plaque sample was placed on the BANA strip. These findings were also related to the presence of *T. denticola* and *P. gingivalis* in the plaque as assessed by an ELISA. The Perioscan results were significantly related to the presence or absence of either *T. denticola* or *P. gingivalis* or both in the plaque samples (Table 6) with a sensitivity of 85%, a specificity of 53%, and an accuracy of 79%. The Perioscan results were also in agreement with the clinical status of the sampled site (Table

TABLE 4. Relationships among Perioscan test, BANA hydrolysis, and spirochete profile of plaque samples

Perioscan result	No. of plaques	BANA hydrolysis (A_{405})[a]	Spirochete profile[a]		
			Total in sample (10^5)	No./hpf	%
Negative	150	0.08 ± 0.009[b]	2.2 ± 0.9[b]	0.1 ± 0.1	5.3 ± 0.8[b]
Weakly positive	16	0.28 ± 0.03[b]	13.2 ± 2.9[b]	0.7 ± 0.4	16.8 ± 2.5
Positive	18	0.51 ± 0.03[b]	28.3 ± 2.8[b]	2.6 ± 0.4[b]	21.8 ± 2.3
ANOVA[c]		$P = 0.0001$	$P = 0.0001$	$P = 0.0001$	$P = 0.0001$

[a] Average ± standard error.
[b] Values significantly different from other values in same column.
[c] Two-factor ANOVA with one random factor being the patient.

TABLE 6. Relationship between Perioscan and ELISA results for both *T. denticola* and *P. (Bacteroides) gingivalis*[a]

Perioscan result	ELISA		Total plaques
	% Negative	% Positive	
Negative	56	15	41
Positive	44	85	139
Total plaques (%)	36 (100)	144 (100)	180

[a] Sensitivity (85%), specificity (53%), and accuracy (79%) estimates were calculated by the correlated binomial model. These differ slightly from values calculated by using the number of sites.

7). If we use the clinical judgment as the reference standard, then the Perioscan test had a sensitivity of 86%, a specificity of 40%, and an accuracy of 74%.

DISCUSSION

The liquid BANA assay has been shown to reliably indicate the presence of high levels of spirochetes (12) and/or *T. denticola* and/or *P. gingivalis* (Bretz et al., in press) in the plaque sample. Since *B. forsythus* is also BANA positive (21), the Perioscan test can give information on this organism. The test will not tell which of these organisms is present, but since they all are anaerobic species, it should enable the clinician to diagnose an anaerobic infection, and such a diagnosis could be useful for the treatment and management of the periodontal disease of the patient. For instance, the liquid BANA test was used to diagnose an anaerobic infection which led to the use of metronidazole and an improvement in the periodontal health of the patient relative to control patients (W. J. Loesche and P. Hujoel, *in* N. W. Johnson, ed., *Markers of Disease Susceptibility and Activity for Periodontal Disease*, in press). Also, a positive BANA test after scaling and root planing was associated with high proportions of spirochetes, suggesting that the treatment rendered was not adequate to suppress or eliminate the anaerobic infection. This was supported by the observation that teeth with BANA-positive plaques at the conclusion of scaling and root planing, as opposed to teeth with BANA-negative plaques, lost significantly more attachment during the year following treatment (W. L. Loesche, J. Giordano, and P. Hujoel, J. Dent. Res. **69**:354, 1990).

This background led us to develop the BANA assay in a format that would be simple, quick, and reliable for use by the dentist at chairside. The present findings indicate that the solid-state paper assays for BANA hydrolysis that have been developed give data comparable to those of the liquid BANA assay. The prototype sandwich method, the Perioscan card format, and the liquid assay were significantly related to the spirochete levels in the plaque (Tables 1 and 4). The liquid and both paper assays gave comparable information relative to the clinical status of the sample tooth site (Tables 2 and 7), with an accuracy of 77% for the liquid BANA, 80% for the sandwich BANA (Table 2), and 74% for the Perioscan BANA (Table 7).

TABLE 7. Relationship between Perioscan BANA results and clinical status of sampled site[a]

Clinical status	Perioscan		Total plaques (%)
	% Negative	% Positive	
Healthy	40	60	47 (100)
Diseased	16	84	133 (100)
Total plaques	40	140	180

[a] Observed proportional agreement of 73% was obtained by jackknife estimation procedures, taking into account the correlated nature of sites (Hujoel et al., in press).

Other diagnostic tests for the periodontal pathogens are being evaluated in various laboratories. An immunofluorescence procedure employing monoclonal antibodies to *P. gingivalis* had an accuracy of 69% when related to the disease status of the patient (22). The detection of *P. gingivalis*, *B. intermedius*, and *A. actinomycetemcomitans* by DNA probes had an accuracy of 69% when related to whether the plaque came from a sample site with a ≥6-mm probing depth (15). The accuracies obtained with all three formats of the BANA assay, i.e., 74 to 80%, are in the same range. However, the criteria for disease assessment in each of these studies were different, so that cross comparisons between the studies may not be valid.

The Perioscan test could be positively associated with the presence of *P. gingivalis* and *T. denticola* in the plaque samples with a sensitivity of 85% (Table 6). The specificity of 53% would indicate that other BANA-positive organisms are present in the plaque. Among the other species tested with the Perioscan, only *B. forsythus* and the *Bacteroides* and *Capnocytophaga* species listed in Materials and Methods were able to give positive or weak reactions. Thus, it is possible that these organisms are present in plaques that are low in or devoid of *T. denticola* and *P. gingivalis* and thereby account for these Perioscan-positive findings.

Note that the in vitro testing of the 60 subgingival species listed in Materials and Methods was weighted to the detection of positive results, i.e., massive numbers of cells were placed on the test strips. The number of cells was at least as large as that obtained by complete removal of plaque from a deep periodontal pocket. Since a plaque sample usually harbors a mixture of species, the number of cells of the species examined in this study should have been as high as, or higher than, that present in plaque samples. Thus, if the in vitro data can be extended to in vivo systems, a positive clinical test is likely to be due to cells of only a limited number of species.

The inconsistent reactions observed for six species deserve additional comment. The reactions differed in terms of their intensities and their consistencies. The presence of large numbers of cells of *T. denticola* or *P. gingivalis* or *B. forsythus* uniformly resulted in large blue areas approximating the site of the test inoculum. The inconsistent reactions, however, were never as large and, when they occurred, were often confined to one margin of the inoculum. The reason for this pattern is not clear, but it could indicate that the BANA-hydrolytic enzyme is not consistently produced by the cells or that the inconsistent species consistently produced this enzyme but at levels which were marginal for the sensitivity of the assay, even when huge masses of cells of that species were inoculated. These considerations suggest that *B. forsythus* and possibly other yet to be identified BANA-positive species, and not the BANA-variable species, were responsible for the Perioscan-positive findings in the absence of detectable *P. gingivalis* and *T. denticola*.

The most apparent difference between the liquid and the paper assays was the higher number of BANA-positive results that were found in the liquid assay (Table 5). This was associated with a higher number of weakly positive results obtained with the liquid assay compared with the paper assay, i.e., 31 versus 16, and suggested that the overnight

incubation period provided enough time for low levels of BANA-positive organisms in the plaque samples to hydrolyze detectable levels of BANA. These low levels can be estimated, with some caution, to be about 10^6 CFU for *T. denticola* and about 10^5 CFU for *P. gingivalis*. The caution reflects the high degree of variability encountered when pure cultures of *T. denticola* and *P. gingivalis* were repeatedly assayed for BANA activity (Table 3). This variability was greatest with *T. denticola*, as indicated by the large standard deviations recorded. These findings indicate that while these species were consistently BANA positive, the level of enzyme activity could be variable, thereby making it difficult to provide definite detection limit values for these organisms in the plaque samples.

The issue of detection limits raises fundamental questions as to how a diagnostic test for various putative periodontal pathogens needs to be designed. Since many of these organisms, such as the spirochetes and *T. denticola* (9, 18), *B. intermedius* (3, 19), *B. forsythus* (3, 5), and *P. gingivalis* (5), can be found in low numbers in many plaque samples in the absence of clinical disease, a diagnostic test(s) with a low detection limit would often yield positive reactions in the presence of clinical health (Loesche and Hujoel, in press). If, however, the presence of the putative pathogen places the individual tooth or patient at risk for periodontitis, as has been suggested for *P. gingivalis* and *A. actinomycetemcomitans* (4, 19), then a test with low detection limits would be preferred.

This issue concerning detection versus a threshold limit for the putative pathogen can be evaluated by examining the relative abilities of sensitive tests (in the bacteriological meaning), like DNA probes and antibodies, and less sensitive tests, like the BANA hydrolysis, to reflect clinical disease. In this regard, we screened young children (average age, 6 years) for the presence of *T. denticola* and/or *P. gingivalis* using the Perioscan assay and ELISA. Most children were detectably colonized with *T. denticola* and/or *P. gingivalis* as determined by the ELISA, but only a limited number were colonized as determined by the BANA assay (M. R. Watson, W. A. Bretz, and W. J. Loesche, J. Dent. Res. **68**, 1989). The level of colonization by BANA-positive organisms but not ELISA-positive organisms could be significantly associated with a history of periodontal disease in a household member. When mothers of BANA-positive and BANA-negative children were examined clinically for periodontal disease, the mothers of the BANA-positive children had significantly more periodontal disease than the mothers of BANA-negative children (M. R. Watson, P. Hujoel, and W. J. Loesche, unpublished data). This suggests that it is more useful to have diagnostic tests that detect certain threshold levels than tests that detect only colonization.

The present findings indicate that a solid-state paper assay for BANA hydrolysis gives data comparable to those obtained with the liquid BANA assay. In addition, the paper assay has certain advantages that would facilitate its clinical use. Thus, a result could be obtained during the same clinical visit in which the plaque sample was taken, thereby enabling the clinician to initiate suitable therapy. Also, since the fast black dye gives a stable color, the Perioscan card could be entered into the permanent treatment file of the patient.

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