Benzoyl-arginine naphthylamide (BANA) hydrolysis by Treponema denticola and/or Bacteroides gingivalis in periodontal plaques


Key words: BANA test; immunofluorescence; Treponema denticola; Bacteroides gingivalis; periodontal plaque

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Treponema denticola (a small spirochete) and Bacteroides gingivalis have been strongly associated with periodontal lesions (7, 8, 18, 19). Both organisms produce a number of virulence factors that may play a role in periodontal tissue destruction, which would include collagenase and lipopolysaccharides (3, 8, 21). Given the importance of these 2 organisms in periodontal disease, rapid and simple assays for monitoring subgingival plaques for these periodontal pathogens would have great clinical value.

T. denticola and B. gingivalis are among the few recognized species found in gingival crevices or periodontal pockets that can hydrolyze the synthetic peptide N-benzoyl-DL-arginine-2-naphthylamide (BANA) (4, 5). It was recently found that Bacteroides forsythus also possesses BANA activity (20). BANA hydrolysis by plaque samples has been significantly associated with increasing probing depth and attachment loss and with high levels and proportions of spirochetes (9). It was not possible to associate BANA hydrolysis with T. denticola, as this organism cannot be quantitatively recovered from plaque samples by cultural procedures (17). The plaque BANA assay was subsequently shown to be a sensitive and specific screening tool for recognizing untreated and treated sites in periodontal patients (15).

In this investigation, we determined the presence of T. denticola and B. gingivalis in BANA-positive or -negative plaque samples, through the use of indirect immunofluorescence techniques employing polyclonal antibodies specific to each organism. Thus, this report focuses on comparisons of BANA hydrolysis with clinical parameters and with the presence and levels of these periodontopathogens in the plaque.

Material and methods

Subgingival plaque samples (2–4 sites per patient) were collected by means of a sterile curette from 17 untreated adult periodontitis patients with no history of antibiotic usage in the past 6 months. A total of 46 samples were collected from 27 diseased sites and 19 healthy sites. The supragingival plaque was removed and discarded, and subgingival specimens were inoculated into a vial containing 0.2 ml of Sorensen phosphate buffer (pH 7.2) and dispersed for 20 s
utilizing a vortex mixer (working suspension).

**Dark-field microscopy**

Ten microliters of the working suspension was placed on a glass slide, covered with a cover slip, sealed, and examined under a dark-field microscope. The total number of bacteria per high-power microscopic field (HPF) and the total number of spirochetes/HPF (which were further subdivided into small, intermediate and large spirochetes) were obtained by counting 200 organisms or the number of organisms in 20 HPF, whichever event occurred first.

**BANA assay**

One hundred microliters of the synthetic substrate n-benzoyl-DL-arginine-2-naphthylamide (BANA), previously diluted (10 µl of 44 mg BANA/ml in DMSO, in 1 ml Sorensen buffer), was added to 100 µl of the working suspension and incubated in aerobic conditions (37°C) for an average of 18 h. The BANA test results were read visually using scoring procedures described elsewhere (1). Separate studies using pure cultures of *B. gingivalis* and *T. denticola* demonstrated that weak-positive results contained about $5 \times 10^2$ colony-forming units (CFU) of *B. gingivalis* and $1 \times 10^6$ CFU of *T. denticola* (10). For purposes of statistical analysis, weak-positive and positive results were grouped as positive results.

**Indirect immunofluorescence assay**

The remaining plaque suspension (90 µl) was added to an equal volume of 1% formaldehyde, yielding a final concentration in the reaction mixture of 0.5% formaldehyde, and stored at 4°C for further analysis. Ten microliters of the formaldehyde-fixed subgingival plaque samples was applied to a 12-well microscope slide, heat-fixed and Gram-stained. These counts provided total bacterial counts per HPF. Subsequently, 10 µl of the plaque samples was applied to a second 12-well slide, heat-fixed and stored at room temperature. Two wells were used for a positive control (pure bacterial culture + antibody + fluorescein isothiocyanate (FITC)-conjugate) and a negative control (pure bacterial culture + buffer + FITC-conjugate).

The plaque samples were stained for 10 min with 10 µl of antisera diluted to working titers in PBS containing 0.05% Tween 20, washed in phosphate-buffered saline (PBS) and rinsed with distilled water. The slides were then incubated with 10 µl of goat anti-rabbit IgG conjugated with FITC. Slides were again rinsed and washed, and subsequently mounted with glycerol in PBS (2:1 v/v) containing para-phenylenediamine (pH 9.0), sealed with a cover slip and nail polish.

Immunofluorescence was evaluated with a Leitz Dialux microscope equipped with a Plomepak 2.3 fluorescence illuminator for epifluorescence. The light source was a 200-W HBO mercury superpressure lamp. Fluorescence was graded from 0 to 4+, with grades 3+ and 4+ considered serologically positive reactions. Staining was considered positive for the selected microorganism(s) if it exhibited strong fluorescent cells with well defined outlines and dark or lightly shining centers, comprising more than 1% of the total cell count. Data were calculated as positive cells per HPF. The antibodies used only stained cells with a spirochetal morphology in the case of the *T. denticola* antibody, or cells with a cocco-bacilli morphology in the case of the *B. gingivalis* antibody, thereby indicating the species specificity of these antibodies.

**Antibodies**

American Type Culture Collection (ATCC) reference strains of *B. gingivalis* (ATCC 33277) and *T. denticola* (ATCC 35405) were grown as previously described (12, 14). The cultures were grown under anaerobic conditions (85% N₂, 10% H₂, and 5% CO₂) at 35°C until maximum turbidity was reached (about 3 to 5 d). The bacteria were harvested by centrifugation and washed 3 times in 0.15 M NaCl. The cells were then resuspended in 1% formaldehyde in PBS and stored at 4°C for 4 d. The cells were then washed 3 times in PBS to remove formaldehyde and lyophilized.

High-titer polyclonal rabbit antibodies were prepared by subcutaneous immunization of female New Zealand
Complete Freund's adjuvant at 7 weeks associated with a routine dental cleaning. Periodontal treatment beyond that as clinical judgement of whether or not on bleeding upon probing (yes or no), Periodontal sites were identified based on the presence of specific species in the plaque. All sera were aliquotted and frozen.

### Clinical parameters of periodontal sites

Diseased (n=27) or healthy (n=19) periodontal sites were identified based on bleeding upon probing (yes or no), probing depth measurements, and the clinical judgement of whether or not that periodontal site should undergo periodontal treatment beyond that associated with a routine dental cleaning by a hygienist.

### Statistical analysis

Frequency tables, 4-fold tables and paired t-tests were calculated using the SAS computer program (SAS Institute, Cary, NC).

### Table 2. Frequency distribution of clinical status, BANA hydrolysis and presence of \( T. denticola \) and/or \( B. gingivalis \) in subgingival plaque samples

<table>
<thead>
<tr>
<th>Presence of species in plaque</th>
<th>Diseased (n=27)</th>
<th>Healthy (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BANA hydrolysis</td>
<td>BANA hydrolysis</td>
</tr>
<tr>
<td></td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>( T. denticola ) yes</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>( T. denticola ) no</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>( B. gingivalis ) yes</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>( B. gingivalis ) no</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Either present</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>Both absent</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

### Results

Optimal working titrations for both \( B. gingivalis \) and \( T. denticola \) antibodies for indirect immunofluorescence were found to be approximately 1/1000. The anti-\( T. denticola \) antibody reacted strongly with the homologous strain, exhibited low cross-reactivity with \( T. vincentii, T. socorbanskii \) and an unspeciated \( T. denticola \), but did not react with the other tested species (Table 1). A score of 2+ denotes a faint fluorescence with poor definition of cell wall. Thus, the cross-reaction observed within spirochetes did not prevent us from recognizing \( T. denticola \) specifically in the plaque sample. The antibodies prepared against \( B. gingivalis \) reacted only with the homologous strains, thereby demonstrating the species specificity of this immunologic reagent (Table 1).

Ninety-five percent of the healthy sites did not bleed upon probing, whereas 89% of the diseased sites bled upon probing. Ninety-five percent of the healthy sites had probing depths ≤3 mm, whereas 100% of the diseased sites had probing depths ≥4 mm. Table 2 shows the frequency of diseased and healthy sites, BANA hydrolysis and immunologic detection of \( T. denticola \) and/or \( B. gingivalis \) in the plaque samples. Eighteen of 27 plaques from diseased sites gave BANA-positive plaques, and 9 plaques from diseased sites gave BANA-negative plaques. Indirect immunofluorescence analysis revealed \( T. denticola \) in 16 of 18 BANA-positive plaques and \( B. gingivalis \) in 9 of 18 BANA-positive plaques. \( T. denticola \) and \( B. gingivalis \) were present in 1 and 2 (respectively) of the 9 BANA-negative plaques. All 19 healthy sites were negative for BANA and \( T. denticola \) and/or \( B. gingivalis \) were not detected.

All measured differences between BANA-positive and BANA-negative plaques obtained in the same individuals were statistically significant (Table 3). BANA-positive plaques harbored \( T. denticola \) at levels of 21 cells/HPF, whereas \( B. gingivalis \) was detected at levels of 3 cells/HPF. Similarly, total number of bacteria/HPF as well as the levels of spirochetes as counted by dark-field microscopy were significantly different between BANA-positive and BANA-negative plaques. BANA-positive reactions were associated with plaques taken from pockets 6.5 mm deep on average, whereas BANA-negative results were associated with plaques taken from shallow pockets of 2.6 mm on average.

The sensitivity and specificity of the BANA test reaction measured against the clinical parameters is shown in Table 4. One would be interested in determining the overall ability of the test, to distinguish between diseased and healthy sites. This can be derived by the sum of true positives plus true negatives divided by the total number of comparisons (accuracy). When the BANA test was measured against the subjectively determined clinical parameters, the accuracy of the BANA test was about 80% (Table 4). The accuracy of the BANA test in terms of detecting the presence of species in plaques (objectively measured parameters) was 93% for \( T. denticola \), 76% for \( B. gingivalis \) and 96% for \( T. denticola \) and/or \( B. gingivalis \) (Table 5).

### Discussion

Several studies have associated \( B. gingivalis \) with periodontal disease status (7, 18, 19, 22) and a few have associated...
B. gingivalis and/or T. denticola positive. (+) = BANA test positive; clinical parameter positive. (-) = BANA test negative; clinical parameter negative. T = total number of observations.

The judgement of a parameter negative. (—) = BANA test negative; clinical parameter positive. (H—) = BANA test positive; clinical parameter positive. ( ) = BANA test negative; clinical parameter negative. ( ) = BANA test positive; clinical parameter negative. (+-) = BANA test positive; clinical parameter positive. (-) = BANA test negative; clinical parameter negative.

False (-) Specificity

Accuracy

Sensitivity

Specificity

False (-)

False (+)

T = total number of observations.

Table 4. Relationship between BANA test and clinical parameters

<table>
<thead>
<tr>
<th>Sensitivity</th>
<th>Clinical judgement</th>
<th>Bleeding upon probing</th>
<th>Probing depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)</td>
<td>66.7</td>
<td>64.0</td>
<td>66.7</td>
</tr>
<tr>
<td>(-)</td>
<td>100.0</td>
<td>90.5</td>
<td>100.0</td>
</tr>
<tr>
<td>(+)</td>
<td>33.3</td>
<td>36.0</td>
<td>33.3</td>
</tr>
<tr>
<td>(-)</td>
<td>0.0</td>
<td>9.5</td>
<td>0.0</td>
</tr>
<tr>
<td>(+)</td>
<td>0.0</td>
<td>9.5</td>
<td>0.0</td>
</tr>
<tr>
<td>(-)</td>
<td>0.0</td>
<td>9.5</td>
<td>0.0</td>
</tr>
</tbody>
</table>

(+) = BANA test positive; clinical parameter positive. (-) = BANA test negative; clinical parameter negative. (+) = BANA test positive; clinical parameter positive. (-) = BANA test negative; clinical parameter negative.

Table 5. Relationship between BANA test and T. denticola and/or B. gingivalis

<table>
<thead>
<tr>
<th>Sensitivity</th>
<th>T. denticola</th>
<th>B. gingivalis</th>
<th>T. denticola and/or B. gingivalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)</td>
<td>94.1</td>
<td>81.9</td>
<td>90.0</td>
</tr>
<tr>
<td>(-)</td>
<td>93.1</td>
<td>74.2</td>
<td>100.0</td>
</tr>
<tr>
<td>(+)</td>
<td>9.5</td>
<td>18.1</td>
<td>10.0</td>
</tr>
<tr>
<td>(-)</td>
<td>6.9</td>
<td>25.8</td>
<td>0.0</td>
</tr>
<tr>
<td>(+)</td>
<td>94.1</td>
<td>76.0</td>
<td>95.7</td>
</tr>
</tbody>
</table>

(+) = BANA test positive; T. denticola and/or B. gingivalis positive. (-) = BANA test negative; T. denticola and/or B. gingivalis negative. (+) = BANA test positive; T. denticola and/or B. gingivalis positive. (-) = BANA test negative; T. denticola and/or B. gingivalis negative. T = total number of observations.

T. denticola (13, 16). This study, however, is the first to use immunofluorescence analysis to associate the frequency and the levels of both T. denticola and B. gingivalis in subgingival plaque samples with periodontal health or disease. Both organisms were present in BANA-positive plaques of periodontally diseased sites, were detected at a low frequency in BANA-negative plaques removed from diseased sites, and were absent from healthy sites that were uniformly BANA-negative (Table 2). The frequency of occurrence of T. denticola in BANA-positive plaques was approximately 2-fold higher than that of B. gingivalis (Table 2) and the levels of T. denticola in BANA-positive plaques were greater than the levels of B. gingivalis (Table 3).

The accuracy of the BANA test vs clinical parameters never exceeded 80% (Table 4), perhaps indicating the subjective nature of the clinical parameters, which are affected by measurement error and examiner variability (6). In this respect, the BANA test may be a more objective measure of identifying periodontal sites potentially at risk for developing periodontal destruction through the detection of periodontal pathogens, such as T. denticola, B. gingivalis and Bacteroides forsythus. Alternatively, the failure to obtain 100% accuracy with the clinical parameters could indicate that, in some plaques, bacteria other than T. denticola and/or B. gingivalis were associated with clinical disease.

The accuracy of the BANA test in detecting T. denticola and/or B. gingivalis was about 96% (Table 5), which means that the BANA test gives information that is as reliable as the information obtained with the immunologic reagents. Given the fact that T. denticola cannot be quantified by cultural methods (17), the BANA test could then be considered as a substitute for both cultural and immunologic methods for detecting T. denticola and possibly B. gingivalis in plaque samples.

The BANA test can detect T. denticola and B. gingivalis when $10^7$ to $10^8$ CFU are present in plaque samples (10). These detection limits make the BANA test less sensitive than other methods, such as DNA probes and cultural procedures, which can detect $10^2$ to $10^3$ CFU. If clinical disease is preceded by or associated with an overgrowth of these periodontal pathogens in plaque samples, then a less sensitive test may be more indicative of an infection that puts periodontal sites at risk for developing periodontal disease.

Several methods, such as cultural, microscopic examination, immunologic reagents, enzyme activity, and DNA probes, could help the clinician monitor plaques for the presence of periodontal pathogens in conjunction with clinical parameters. The BANA test offers the potential to identify plaques that may harbor T. denticola, B. gingivalis and a third organism, B. forsythus, which has recently been associated with the progressing periodontal lesion (2). The BANA test would then be a simple method of detecting periodontal anaerobic infections.

In summary, this study has indicated that: BANA-positive plaques are associated with the presence of T. denticola and/or B. gingivalis; T. denticola was found at greater frequency and levels in BANA-positive plaques than was B. gingivalis; and the presence of the organisms is associated with clinical inflammation in deep periodontal pockets. A BANA test in a solid state that can be performed at chairside has been recently evaluated and shows an accuracy of 84% with B. gingivalis and T. denticola (11).

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References

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