Antibiotypes of *Bacteroides gingivalis* Assessed by Antimicrobial Disks and Multivariate Analysis

F. J. W. NOTTEN, F. H. M. NIEMAN, AND F. H. M. MIKX*

Department of Preventive and Community Dentistry, University of Nijmegen, Nijmegen, The Netherlands

Received 29 April 1985/Accepted 5 September 1985

Antibiograms with 20 different antimicrobial disks were studied for antibiotyping of *Bacteroides gingivalis* isolates. The stability of the antibiotypes was tested by passage in mice. Several *B. gingivalis* isolates of the same subject were used to investigate the presence of different antibiotypes in one individual, while isolates from different subjects were used to investigate individual differences. The antibiotypes were found to remain stable after animal passage. All tested strains of different origin represented different antibiotypes. The isolates from one subject all belonged to the same antibiotype. Principal component analysis of the data showed that two factors were important in the discrimination of the strains of *B. gingivalis*. One included β-lactam antimicrobial agents that affect the cell wall. The other included antimicrobial agents that inhibit synthesis of protein and nucleic acid. Both principal component analysis and discriminant analysis proved to be of great use in the reduction of the amount of data and the visualization of the relations between different antibiotypes of *B. gingivalis* in a linear map. Among the investigated subjects, different antibiotypes of *B. gingivalis* were found, indicating that in the mouth of an individual, one antibiotype of *B. gingivalis* predominates and that different persons harbor different antibiotypes of *B. gingivalis*.

*Bacteroides gingivalis* appears to be one of the most virulent microorganisms in relation to periodontal disease (11, 12). Until now, little was known about the acquisition and distribution of *B. gingivalis* among periodontal patients, in contrast to another oral pathogen, *Streptococcus mutans*. Rogers (9) found that one type of *S. mutans* predominates in the mouth of an individual and that intrafamilial transmission of *S. mutans* occurs, especially from the mother to the child. In addition, Zambron et al. (14) found that patients with juvenile periodontitis harbored the same biotype and serotype of *Actinobacillus actinomycetemcomitans* as did their families.

So far, biotyping and serotyping of *B. gingivalis* is not available. Other methods to identify individual strains of microorganisms are antibiotyping and typing by mass spectrometry of volatile pyrolysis products (1, 2). Borst et al. (2) described a method for typing microorganisms by using antimicrobial agent-containing disks placed on seeded agar plates. After growth, the diameters of the inhibition zones were measured. However, the analysis was done by visual comparison of the data. The pattern of inhibition zones appeared to be specific for each strain. In a pilot study, this method was applied for *B. gingivalis* (7). In the present study, the processing of the data was done by more advanced statistical methods. These methods were used as a means to identify different types of *B. gingivalis* in individual patients.

**MATERIALS AND METHODS**

**Bacterial strains.** Seven strains of *B. gingivalis* were tested. Strains OMB 8005-8, OMB 2901.06, OMB 11054, and OMB 12301 were provided by F. Gusberti, Klinik Für Kronen-Brückenprothetik, University of Bern, Bern, Switzerland. Strains Ny 467, Ny 467-1, Ny 467-2, Ny 467-3, Ny 468, and Ny 469 were isolated by M. A. C. van Oosten in the Department of Periodontology of the University of Nijmegen, Nijmegen, The Netherlands. The strains were isolated from different patients with periodontitis.

**Cultivation.** *B. gingivalis* was cultured in the BM broth of Gibbons and MacDonald (3) supplemented with 0.25% liver digest (Oxoid Ltd., London, United Kingdom) or on BM agar with 0.25% liver digest and 5% defibrinated sheep blood (8). All culturing and dilution procedures were performed on prereduced media in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) at 37°C in an 85% N2–10% CO2–5% H2 atmosphere, unless indicated otherwise. The *B. gingivalis* isolates were stored at –80°C after addition of 7% (vol/vol) dimethylsulfoxide to 24-h cultures in BM broth.

**Inoculation of mice.** To study the stability of the antibiogram, *B. gingivalis* Ny 467 and Ny 469 were given an animal passage, as described by van Steenbergen et al. (13). Inocula (50 μl) of both strains, containing approximately 10⁸ cells, were injected subcutaneously in the back of five 4-week-old male Swiss mice. Up to 10 days after inoculation, samples were taken from the lesions and cultured on BM agar under anaerobic conditions. Black-pigmented colonies were isolated and classified according to characteristic biochemical properties, including trypsin activity, fermentation of glucose, production of indole, esculin hydrolysis, catalase activity, and hemagglutination (5).

**Antibiotyping.** With some modifications, the antibiotyping was carried out as described by Borst et al. (2). The following 20 antimicrobial disks were selected: ampicillin (AM10), carbenicillin (CB50), cefazolin (CZ30), methicillin (PD5), penicillin (P2), chloramphenicol (C30), clindamycin (CC2), erythromycin (E2), furazolidone (Fx100), fusidin acid (FA10), nitrofurantoin (FM100), oxytetracycline (T30), and tetracycline (Te5), delivered by Becton Dickinson and Co., Paramus, N.J.; cephaloridine (CR5), bacitracin (B10), minocycline (MF30), spectinomycin (Si30), rifampin (RD2), and novobiocin (NV5), delivered by Oxoid Ltd., Basingstoke, England; and metronidazole (M25) from May & Baker Ltd., Dagenham, England. In the preparation of the seeded agar plates, 160 ml of BM agar (0.75% Noble agar; Difco Laboratories, Detroit, Mich.) was mixed at 40°C with 40 ml of a
24-h broth culture of the B. gingivalis test strain. Portions (6 ml) of the seeded agar were poured into 9-cm petri dishes. After cooling, the plates were transported into the anaerobic chamber, and one antimicrobial disk was placed on each plate. The whole procedure from preparation to incubation was performed within 30 min. After incubation for 2 days, the diameters of the zones of inhibition were measured in millimeters.

Processing of data. Antibiograms provided data that vary by measurement and strain (see Table 1). The measurement characteristics of the data permitted the use of such statistics as means, correlations, and variances. The total variance formed the basis for further analysis. The total variance of the antibiograms is the sum of the individual variances within the strains and variances between the strains. In a correlation matrix, the coherence of the different antibiotics in the antibiograms was calculated (see Table 2). To reduce the illegible mass of correlations, the basis of these correlations was searched for by the principal component analysis, a form of factor analysis (see Table 3). From the results of this analysis, a two-dimensional scattergram was constructed using the two factors found by

---

### TABLE 1. Antibiogram of seven B. gingivalis strains

<table>
<thead>
<tr>
<th>Antimicrobial disk</th>
<th>Ny 467</th>
<th>Ny 468</th>
<th>Ny 469</th>
<th>OMB 8005-8</th>
<th>OMB 2901.06</th>
<th>OMB 11054</th>
<th>OMB 12301</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>56.7 ± 1.2</td>
<td>69.3 ± 2.1</td>
<td>42.7 ± 1.5</td>
<td>69.7 ± 2.5</td>
<td>49.0 ± 1.0</td>
<td>69.0 ± 1.7</td>
<td>56.7 ± 4.5</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>27.3 ± 1.5</td>
<td>28.7 ± 6.8</td>
<td>34.3 ± 0.6</td>
<td>47.0 ± 2.7</td>
<td>27.0 ± 1.0</td>
<td>38.0 ± 2.5</td>
<td>32.3 ± 2.3</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>67.0 ± 2.8</td>
<td>74.0 ± 1.4</td>
<td>49.0 ± 1.4</td>
<td>75.0 ± 0.0</td>
<td>52.5 ± 3.5</td>
<td>75.0 ± 0.0</td>
<td>61.3 ± 2.3</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>48.0 ± 2.7</td>
<td>61.7 ± 3.2</td>
<td>35.7 ± 2.1</td>
<td>69.3 ± 2.1</td>
<td>41.7 ± 2.5</td>
<td>63.3 ± 1.2</td>
<td>50.0 ± 2.6</td>
</tr>
<tr>
<td>Cephlorodin</td>
<td>50.7 ± 1.2</td>
<td>62.3 ± 2.1</td>
<td>39.0 ± 0.0</td>
<td>67.3 ± 4.2</td>
<td>43.0 ± 2.7</td>
<td>64.7 ± 1.5</td>
<td>51.0 ± 1.7</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>32.3 ± 3.1</td>
<td>42.7 ± 2.1</td>
<td>31.0 ± 1.5</td>
<td>59.3 ± 10.1</td>
<td>32.0 ± 1.0</td>
<td>41.0 ± 4.4</td>
<td>32.7 ± 1.2</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>54.0 ± 2.0</td>
<td>62.7 ± 3.5</td>
<td>40.0 ± 1.6</td>
<td>74.3 ± 0.6</td>
<td>42.3 ± 2.5</td>
<td>54.7 ± 5.1</td>
<td>44.0 ± 2.7</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>37.0 ± 1.7</td>
<td>44.0 ± 1.7</td>
<td>28.7 ± 1.5</td>
<td>52.7 ± 1.5</td>
<td>31.3 ± 2.1</td>
<td>49.1 ± 2.3</td>
<td>41.7 ± 1.2</td>
</tr>
<tr>
<td>Furazolidone</td>
<td>31.3 ± 2.9</td>
<td>34.0 ± 6.3</td>
<td>19.0 ± 1.0</td>
<td>56.0 ± 8.0</td>
<td>18.3 ± 1.2</td>
<td>29.7 ± 3.1</td>
<td>20.0 ± 1.0</td>
</tr>
<tr>
<td>Fusidin acid</td>
<td>47.7 ± 3.5</td>
<td>60.7 ± 1.5</td>
<td>38.3 ± 0.6</td>
<td>73.0 ± 1.7</td>
<td>39.3 ± 2.1</td>
<td>51.7 ± 3.2</td>
<td>41.7 ± 0.6</td>
</tr>
<tr>
<td>Methicillin</td>
<td>24.0 ± 2.7</td>
<td>41.0 ± 2.0</td>
<td>21.7 ± 2.1</td>
<td>54.0 ± 10.2</td>
<td>22.7 ± 0.6</td>
<td>49.7 ± 1.5</td>
<td>27.0 ± 1.0</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>48.7 ± 1.5</td>
<td>50.7 ± 12.0</td>
<td>28.0 ± 3.6</td>
<td>71.7 ± 4.9</td>
<td>37.0 ± 13.2</td>
<td>50.0 ± 6.6</td>
<td>33.7 ± 4.2</td>
</tr>
<tr>
<td>Minocycline</td>
<td>48.7 ± 2.3</td>
<td>56.7 ± 2.5</td>
<td>35.3 ± 0.6</td>
<td>73.3 ± 1.5</td>
<td>41.3 ± 3.1</td>
<td>50.0 ± 3.3</td>
<td>39.3 ± 2.1</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>44.0 ± 3.5</td>
<td>15.3 ± 6.1</td>
<td>31.0 ± 0.8</td>
<td>59.0 ± 3.6</td>
<td>27.3 ± 2.5</td>
<td>40.7 ± 2.5</td>
<td>29.7 ± 2.3</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>20.7 ± 1.5</td>
<td>48.7 ± 1.2</td>
<td>27.3 ± 0.6</td>
<td>64.3 ± 0.6</td>
<td>41.7 ± 0.6</td>
<td>45.7 ± 2.3</td>
<td>33.7 ± 1.2</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>45.7 ± 1.5</td>
<td>55.7 ± 2.9</td>
<td>34.3 ± 1.2</td>
<td>70.3 ± 4.7</td>
<td>39.7 ± 1.2</td>
<td>55.0 ± 4.4</td>
<td>41.0 ± 1.7</td>
</tr>
<tr>
<td>Penicillin</td>
<td>53.3 ± 2.3</td>
<td>62.3 ± 0.6</td>
<td>38.3 ± 1.5</td>
<td>67.0 ± 2.7</td>
<td>42.0 ± 2.7</td>
<td>63.0 ± 2.0</td>
<td>49.0 ± 1.5</td>
</tr>
<tr>
<td>Rifampin</td>
<td>41.0 ± 1.7</td>
<td>50.3 ± 1.2</td>
<td>25.0 ± 2.7</td>
<td>63.0 ± 10.5</td>
<td>39.7 ± 1.2</td>
<td>38.0 ± 1.0</td>
<td>36.3 ± 0.6</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>24.0 ± 2.0</td>
<td>30.7 ± 2.1</td>
<td>16.7 ± 1.2</td>
<td>30.3 ± 10.5</td>
<td>19.7 ± 0.6</td>
<td>25.7 ± 3.8</td>
<td>19.3 ± 0.6</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>42.0 ± 1.7</td>
<td>51.0 ± 3.0</td>
<td>32.0 ± 1.0</td>
<td>58.7 ± 4.0</td>
<td>35.7 ± 1.5</td>
<td>52.6 ± 3.5</td>
<td>32.3 ± 3.1</td>
</tr>
</tbody>
</table>

* Each value represents the mean ± standard deviation of results from three identical tests.

---

### TABLE 2. Correlation matrix of 14 antimicrobial agents

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Factor 1</th>
<th>Carbenicillin</th>
<th>Cefazolin</th>
<th>Cephlorodin</th>
<th>Methicillin</th>
<th>Penicillin</th>
<th>Clindamycin</th>
<th>Furazolidone</th>
<th>Fusidin acid</th>
<th>Metronidazole</th>
<th>Minocycline</th>
<th>Nitrofurantoin</th>
<th>Rifampin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Factor 1</strong></td>
<td>1.00</td>
<td>0.97</td>
<td>0.96</td>
<td>0.97</td>
<td>0.87</td>
<td>0.98</td>
<td>0.73</td>
<td>0.83</td>
<td>0.83</td>
<td>0.74</td>
<td>0.79</td>
<td>0.73</td>
<td>0.71</td>
</tr>
<tr>
<td><strong>Factor 2</strong></td>
<td></td>
<td>0.97</td>
<td>0.94</td>
<td>0.96</td>
<td>0.96</td>
<td>0.98</td>
<td>0.97</td>
<td>0.94</td>
<td>0.94</td>
<td>0.88</td>
<td>0.91</td>
<td>0.90</td>
<td>0.71</td>
</tr>
</tbody>
</table>

* Results were obtained from biotype of seven B. gingivalis strains and are grouped on the basis of factor analysis results.

* Mean correlations: between factor 1 antimicrobial agents, 0.94; between factor 2 antimicrobial agents, 0.90; between factor 1 and factor 2 antimicrobial agents, 0.79.
the factor analysis. In this scattergram, the positions of the 
antibiograms of every strain could be indicated, by the use of 
the mean score of inhibition zones of the antimicrobial 
agents belonging to factor 1 on the x axis and those belonging 
to factor 2 on the y axis. Connection of the points of the three 
antibiograms of each strain in the scattergram gave the 
encouraging impression that the variances within each factor 
group were relatively small compared with the variances 
between groups. To test this impression, a statistical tech-
nique was needed to analyze the variance between groups. 
Discriminant analysis was used to test whether the mean 
values of the antibiograms of the strains were mutually 
different in reality (4). The principal component analysis and 
the discriminant analysis are programs in the SPSS, Statis-
tical Package of Social Sciences (6).

RESULTS

B. gingivalis isolates of seven different subjects were 
tested. The reproducibility of the antibiograms tested in 
triplicate is shown in Table 1. It was found that different 
strains had different sensitivities. In a pilot study, principal 
component analysis showed that of the 20 antimicrobial 
agents tested, 14 were relevant in the discrimination of the B. 

ingivalis strains. The correlations between these 14 anti-
microbial agents were calculated (Table 2). Highly correlated 
were the β-lactams (mean correlation, 0.94). Also highly 
correlated were the antibiotics that inhibit the synthesis of 
protein or nucleic acid (mean correlation, 0.90), with the 
exception of rifampin (mean correlation, 0.83). The mean 
correlation between the β-lactams and the other antimicro-
bial agents was 0.79.

Two main factors were extracted by the principal com-
ponent analysis program (Table 3). Both factors together 
account for 93.3% of total variance. The principal compo-
nent matrix showed that the first factor contains β-lactam 
while the antimicrobial agents that inhibit the synthesis of 
proteins or nucleic acids have high factor loadings on the 
second factor. The relative positions of triplicates of each 
strain on both factors were calculated by dividing the sum of 
the inhibition zone diameters by the number of relevant 
antimicrobial agents. The triplicates were then placed in a 
two-dimensional scattergram (Fig. 1). The scattergram plot 
provided by its two dimensions the basis for the assumption 
that each of the seven strains clusters apart from the others. 
The statistical evidence for this assumption was given by the 
subsequent discriminant analysis, which demonstrated that 
the relative distances between the seven strains were indeed 
statistically large enough to reject the null hypothesis that all 
(or even some) triplicates belonged to the same group. A 
100% classification success was achieved by cross-tabulating 
the hypothetical group classification with the predicted 
group classification based on the predictive power of a linear 
set of the 14 antimicrobial agents. Further discriminant 
analysis showed that all tested strains of B. gingivalis could 
have been identified correctly with only four antimicrobial 
agents if the agents were chosen from both factors.

Samples were obtained several times from the subject 
harboring B. gingivalis Ny 467, and three more isolates were 
collected. B. gingivalis Ny 467 and Ny 467-1 were collected 
from the same site, but with a time interval of 6 months. 
Isolates Ny 467-2 and Ny 467-3 were collected from another 
site, also with a time interval of 6 months.

The scattergram of the in duplo-tested strains (Fig. 2) 
provided the basis for the assumption that every isolate had 
an antibiotype that clearly differed from the antibiotype of 
the reference strain Ny 469. Several runs with the SPSS 
program demonstrated that the relative distances between 
strain Ny 469 and strains Ny 467, Ny 467-1, Ny 467-2, and 
Ny 467-3 were statistically large enough to reject the null 
hypothesis. This indicated that the isolates of B. gingivalis of 
one patient were clearly different from the B. gingivalis Ny 
469 of another patient. Cross-tabulation of these strains 
showed a 100% classification success.

The stability of the antibiograms of B. gingivalis NY 467 
and Ny 469 was tested in an animal model. After subcuta-
aneous injection of 10 mice with these strains, gravity ab-
scesses developed at the abdominal area. From these ab-
scesses, samples were taken up to 10 days after injection. 
Eleven isolates were recovered from mice infected with B. 
gingivalis Ny 467, and six isolates were recovered from mice 
infected with strain Ny 469. Antibiograms of all strains,

<p>| TABLE 3. Factor loadings in the principal component analysis of 14 antimicrobial agents* |
|-----------------------------------------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Factor 1</th>
<th>Factor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>0.90</td>
<td>0.40</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>0.86</td>
<td>0.46</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>0.83</td>
<td>0.53</td>
</tr>
<tr>
<td>Cephaloridin</td>
<td>0.89</td>
<td>0.45</td>
</tr>
<tr>
<td>Methicillin</td>
<td>0.79</td>
<td>0.47</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.85</td>
<td>0.51</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0.39</td>
<td>0.87</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.56</td>
<td>0.81</td>
</tr>
<tr>
<td>Furazolidone</td>
<td>0.38</td>
<td>0.90</td>
</tr>
<tr>
<td>Fusidin acid</td>
<td>0.57</td>
<td>0.80</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>0.48</td>
<td>0.80</td>
</tr>
<tr>
<td>Minocycline</td>
<td>0.52</td>
<td>0.83</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>0.44</td>
<td>0.85</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.47</td>
<td>0.74</td>
</tr>
</tbody>
</table>

* Results are derived from antibiotyping of the seven B. gingivalis strains
b Factor loading indicates the relative weight of the individual antibiotic on each of the two factors. Factors 1 and 2 account for 93.3% of the explained variance.
including the parent strains, were made and analyzed with the principal component analysis program. The results are presented in a two-dimensional scattergram (Fig. 3). The isolates after animal passage of strains Ny 467 and Ny 469 showed some differences. However, every isolate clustered with its corresponding parent strain. Additional discriminant analysis showed that the two clusters were classified 100% correctly.

DISCUSSION

Rapid growth is a prerequisite for the presented antibiotyping method (10). All B. gingivalis strains were cultured under standardized conditions and reached the stationary phase in BM broth within 24 h. Under these conditions, the antibiograms of B. gingivalis were shown to be reproducible and stable after animal passage.

The number of antimicrobial agents in an antibiogram is defined by the discriminating properties of the antibiotics and the level of discrimination wanted. The selection of 20 antimicrobial agents for typing of B. gingivalis was based upon the antibiotyping results of Borst et al. (2) with Pseudomonas sp. and upon a pilot study with B. gingivalis (7). In the present study, 14 antimicrobial agents were shown to be important in the discrimination of the B. gingivalis strains. In this discrimination, two factors were important. One factor included the β-lactam antibiotics; the other factor included the inhibition of protein or nucleic acid synthesis.

The two factors in the antibiotyping were highly correlated. For example, B. gingivalis OMB 8005-8 was very susceptible not only to β-lactam antibiotics but also to antimicrobial agents inhibiting the synthesis of protein or nucleic acid. It is speculated that the permeability of the bacterial cell wall underlies this correlation.

In antibiotyping of B. gingivalis, the statistical analyses proved to be of great use. The multivariate analysis techniques reduced the amount of data and visualized the relations between different antibiotypes of B. gingivalis by a linear map. In addition, the discriminant analysis showed that the B. gingivalis strains could have been identified correctly with only four antimicrobial agents if the agents were chosen from both factors.

The present findings indicate that in the mouth of an individual, one antibiotype of B. gingivalis predominates and that different patients harbor different antibiotypes of B. gingivalis. Similar results were found by Rogers (9) with S. mutans and by Zambon et al. (14) with A. actinomycetemcomitans. The occurrence of different types of B. gingivalis might make possible the study of the acquisition and transfer of this periodontal pathogen and raises questions about the biological background of this phenomenon.

ACKNOWLEDGMENTS

We thank N. P. Lang, F. Gusberti, and M. A. C. van Oosten for their cooperation in the project and M. van den Boogaard for typing the manuscript.

LITERATURE CITED


