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

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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, Zambon 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, andNy 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% N_2–10% CO_2–5% H_2 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 10^9 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 (F/M100), oxytetracycline (T30), and tetracycline (Te5), delivered by Becton Dickinson and Co., Paramus, N.J.; cephaloridin (CR5), bacitracin (B10), minocycline (MH30), 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

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

Antimicrobial disk	Diameter of inhibition zone (mm) with the following *B. gingivalis* strain[a]:						
	Ny 467	Ny 468	Ny 469	OMB 8005-8	OMB 2901.06	OMB 11054	OMB 12301
Ampicillin	56.7 ± 1.2	69.3 ± 2.1	42.7 ± 1.5	69.7 ± 2.5	49.0 ± 1.0	69.0 ± 1.7	56.7 ± 4.5
Bacitracin	27.3 ± 1.5	28.7 ± 6.8	34.3 ± 0.6	47.0 ± 2.7	27.0 ± 1.0	38.0 ± 2.5	32.3 ± 2.3
Carbenicillin	67.0 ± 2.8	74.0 ± 1.4	49.0 ± 1.4	75.0 ± 0.0	52.5 ± 3.5	75.0 ± 0.0	61.3 ± 2.3
Cefazolin	48.0 ± 2.7	61.7 ± 3.2	35.7 ± 2.1	69.3 ± 2.1	41.7 ± 2.5	61.3 ± 1.2	50.0 ± 2.6
Cephaloridin	50.7 ± 1.2	62.3 ± 2.1	39.0 ± 0.0	67.3 ± 4.2	43.0 ± 2.7	64.7 ± 1.5	51.0 ± 1.7
Chloramphenicol	32.3 ± 3.1	42.7 ± 2.1	31.0 ± 1.5	59.3 ± 10.1	32.0 ± 1.0	41.0 ± 4.4	32.7 ± 1.2
Clindamycin	54.0 ± 2.0	62.7 ± 3.5	40.0 ± 1.6	74.3 ± 0.6	42.3 ± 2.5	54.7 ± 5.1	44.0 ± 2.7
Erythromycin	37.0 ± 1.7	44.0 ± 1.7	28.7 ± 1.5	52.7 ± 1.5	31.3 ± 2.1	49.1 ± 2.3	41.7 ± 1.2
Furazolidone	31.3 ± 2.9	34.0 ± 6.3	19.0 ± 1.0	56.0 ± 8.0	18.3 ± 1.2	29.7 ± 3.1	20.0 ± 1.0
Fusidin acid	47.7 ± 3.5	60.7 ± 1.5	38.3 ± 0.6	73.0 ± 1.7	39.3 ± 2.1	51.7 ± 3.2	41.7 ± 0.6
Methicillin	24.0 ± 2.7	41.0 ± 2.0	21.7 ± 2.1	54.0 ± 10.2	22.7 ± 0.6	49.7 ± 1.5	27.0 ± 1.0
Metronidazole	48.7 ± 1.5	50.7 ± 12.0	28.0 ± 3.6	71.7 ± 4.9	37.0 ± 13.2	50.0 ± 6.6	33.7 ± 4.2
Minocycline	48.7 ± 2.3	56.7 ± 2.5	35.3 ± 0.6	73.3 ± 1.5	41.3 ± 3.1	50.0 ± 5.3	39.3 ± 2.1
Nitrofurantoin	44.0 ± 3.5	15.3 ± 6.1	31.0 ± 0.8	59.0 ± 3.6	27.3 ± 2.5	40.7 ± 2.5	29.7 ± 2.3
Novobiocin	20.7 ± 1.5	48.7 ± 1.2	27.3 ± 0.6	64.3 ± 0.6	41.7 ± 0.6	45.7 ± 2.3	33.7 ± 1.2
Oxytetracycline	45.7 ± 1.5	55.7 ± 2.9	34.3 ± 1.2	70.3 ± 4.7	39.7 ± 1.2	55.0 ± 4.4	41.0 ± 1.7
Penicillin	53.3 ± 2.3	62.3 ± 0.6	38.3 ± 1.5	67.0 ± 2.7	42.0 ± 2.7	63.0 ± 2.0	49.0 ± 1.5
Rifampin	41.0 ± 1.7	50.3 ± 1.2	25.0 ± 2.7	63.0 ± 10.5	39.7 ± 1.2	38.0 ± 1.0	36.3 ± 0.6
Spectinomycin	24.0 ± 2.0	30.7 ± 2.1	16.7 ± 1.2	30.3 ± 10.5	19.7 ± 0.6	25.7 ± 3.8	19.3 ± 0.6
Tetracycline	42.0 ± 1.7	51.0 ± 3.0	32.0 ± 1.0	58.7 ± 4.0	35.7 ± 1.5	52.6 ± 3.5	32.3 ± 3.1

[a] Each value represents the mean ± standard deviation of results from three identical tests.

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 2. Correlation matrix of 14 antimicrobial agents[a]

Antimicrobial agent	Correlation with[b]:													
	Factor 1						Factor 2							
	Ampicillin	Carbenicillin	Cefazolin	Cefaloridin	Methicillin	Penicillin	Chloramphenicol	Clindamycin	Furazolidone	Fusidin acid	Metronidazole	Minocycline	Nitrofurantoin	Rifampin
Factor 1														
Ampicillin	1.00													
Carbenicillin	0.97	1.00												
Cefazolin	0.96	0.94	1.00											
Cephaloridin	0.97	0.96	0.98	1.00										
Methicillin	0.87	0.82	0.90	0.93	1.00									
Penicillin	0.98	0.98	0.96	0.98	0.89	1.00								
Factor 2														
Chloramphenicol	0.73	0.69	0.79	0.73	0.76	0.79	1.00							
Clindamycin	0.83	0.88	0.89	0.86	0.81	0.89	0.90	1.00						
Furazolidone	0.71	0.74	0.80	0.74	0.73	0.79	0.97	0.93	1.00					
Fusidin acid	0.83	0.83	0.90	0.86	0.84	0.88	0.92	0.98	0.94	1.00				
Metronidazole	0.74	0.83	0.81	0.78	0.75	0.79	0.86	0.92	0.88	0.86	1.00			
Minocycline	0.79	0.83	0.88	0.84	0.81	0.86	0.91	0.97	0.93	0.98	0.89	1.00		
Nitrofurantoin	0.73	0.82	0.88	0.77	0.71	0.82	0.90	0.95	0.96	0.93	0.88	0.92	1.00	
Rifampin	0.71	0.71	0.80	0.76	0.72	0.75	0.75	0.88	0.78	0.87	0.83	0.91	0.76	1.00

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

[b] 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.

TABLE 3. Factor loadings in the principal component analysis of 14 antimicrobial agents[a]

Antimicrobial agent	Factor loading[b]:	
	Factor 1	Factor 2
Ampicillin	0.90	0.40
Carbenicillin	0.86	0.46
Cefazolin	0.83	0.53
Cephaloridin	0.89	0.45
Methicillin	0.79	0.47
Penicillin	0.85	0.51
Chloramphenicol	0.39	0.87
Clindamycin	0.56	0.81
Furazolidone	0.38	0.90
Fusidin acid	0.57	0.80
Metronidazole	0.48	0.80
Minocycline	0.52	0.83
Nitrofurantoin	0.44	0.85
Rifampin	0.47	0.74

[a] 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.

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 technique 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, Statistical 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. gingivalis* strains. The correlations between these 14 antimicrobial 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 antimicrobial agents was 0.79.

Two main factors were extracted by the principal component analysis program (Table 3). Both factors together account for 93.3% of total variance. The principal component 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 subcutaneous injection of 10 mice with these strains, gravity abscesses developed at the abdominal area. From these abscesses, 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,

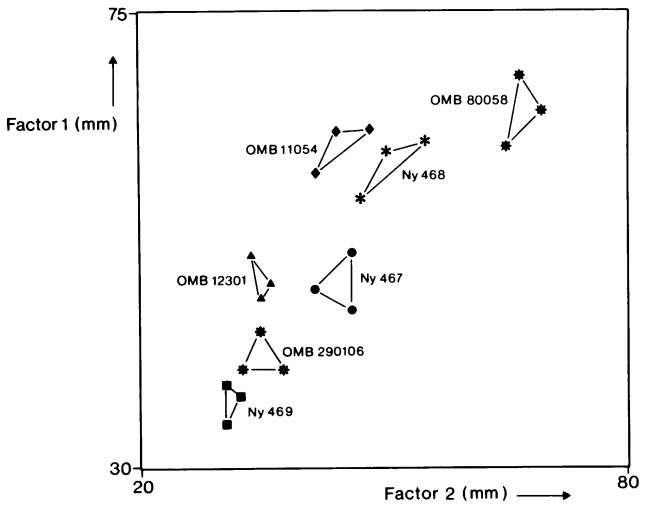

FIG. 1. Scattergram of seven strains of *B. gingivalis*. The values on the axis indicate the mean sum of inhibition zones, in millimeters, of the antimicrobial agents belonging to the two factors. Factor 1 includes β-lactam antibiotics. Factor 2 includes antimicrobial agents that inhibit the synthesis of protein and nucleic acid. All strains were tested in triplicate.

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.

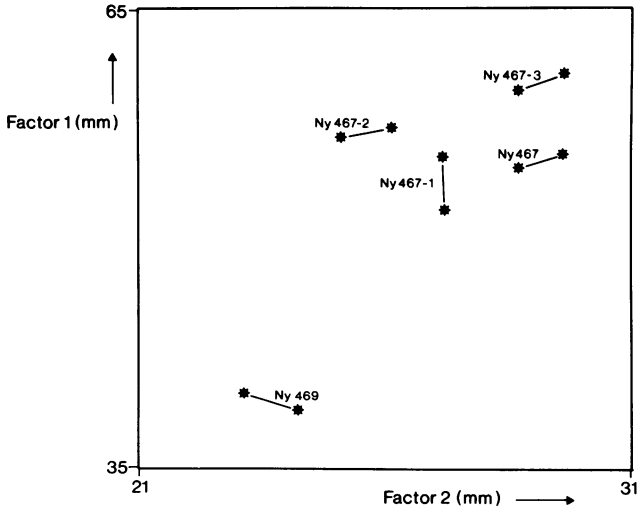

FIG. 2. Scattergram of a reference strain of *B. gingivalis* Ny 469 and four strains from one subject, Ny 467, Ny 467-1, Ny 467-2 and Ny 467-3, isolated at two different sites in a time interval of 6 months. All strains were tested twice. Axes are as described in the legend to Fig. 1.

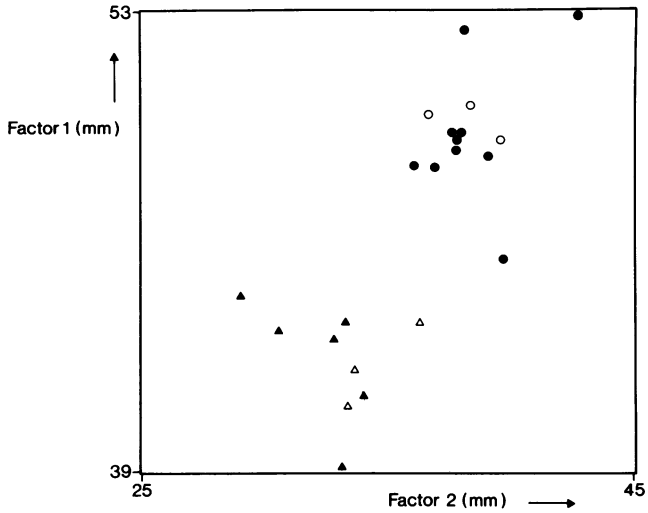

FIG. 3. Scattergram of *B. gingivalis* Ny 467 (○, ●) and Ny 469 (△, ▲). Open symbols indicate parent strains; closed symbols indicate strains after animal passage. The parent strains were tested in triplicate.

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.

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