Antigenic Variation in *Bacteroides forsythus* Detected by a Checkerboard Enzyme-Linked Immunosorbent Assay

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Evidence indicating that multiple serotypes of *Bacteroides forsythus* participate in rapidly progressing periodontal infections has not been reported previously. Our aim was to develop an assay for detecting subsets of *B. forsythus* clinical isolates which differ in serogroup membership and subsets of patients with immunoglobulin G (IgG) responses which differ in serogroup recognition. A checkerboard enzyme-linked immunosorbent assay (ELISA) was used to assess variation in the IgG binding profiles of 22 clinical isolates in sera from 28 patients with early-onset rapidly progressive periodontitis. To accommodate the maximum number of isolates and sera in a given assay run, a multiplate assay grid with standard 96-well microtest plates was established. Single dilutions of individual sera were placed in rows crossing columns of isolate-coated wells, and antigen-specific IgG immobilized in the wells was measured as ELISA absorbance. Pooled sera and isolates were assayed in parallel to serve as negative controls for variation in IgG binding profiles. Correlation and hierarchical cluster analysis of the absorbance data matrix showed that the isolates could be sorted into at least four clusters based on variations in their IgG binding profiles across different sera. Furthermore, at least two patient clusters were defined by variations in their serum IgG antigen recognition profiles across different isolates. We conclude that multiple serogroups of *B. forsythus* exist and that different serogroups are dominant in the antibody response of different patients. The method applied here could be used to serologically classify clinical isolates of other species which evoke a serum antibody response in patients.

Periodontitis is an immunoinflammatory response of susceptible individuals to subgingival microbial plaque in which tissues supporting the teeth are destroyed. The consensus finding of the American Academy of Periodontology 1996 World Workshop in Clinical Periodontics was that sufficient data exist to consider at least three gram-negative plaque species key etiologic agents in destructive periodontal diseases: *Bacteroides forsythus*, *Porphyromonas gingivalis*, and *Actinobacillus actino-

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nosorbert assay (CELISA) system which relies on correlation and hierarchical cluster analysis to reveal subsets of clinical isolates which differ in serogroup membership and subsets of patients with immunoglobulin G (IgG) responses dominant for different serogroups. The new method can easily be adapted for the serological classification of bacterial pathogens associated with other types of infections.

## MATERIALS AND METHODS

### Serum samples

Sera were obtained from patients diagnosed with early-onset rapidly progressive periodontitis (n = 28) prior to treatment at the University of Washington Graduate Periodontics Clinic or in the private practice of one of the authors (R.C.P.). Fifteen of the patients were female (1 oral and 14 white) and 13 were male (1 black, 1 oriental, and 11 white). The mean age of the serum donors was 34 years. All test sera were diluted 1:400 in blocking buffer for use in the CELISA test panel based on prescreening data (see Results).

### Bacterial isolates

*B. forsythus* 43057 was obtained from the American Type Culture Collection (ATCC), and 21 oral isolates were also obtained from patients diagnosed as having early-onset rapidly progressive periodontitis. All isolates were taken from gingival lesions of patients by cuvette or paper point sampling prior to treatment in the above mentioned clinics. All isolates were cultured anaerobically on heart infusion agar and grown in heart infusion broth supplemented with pooled sera and subcultured heterologous relative to the sera included in the study. Isolates were subcultured prior to treatment in the above mentioned clinics. All isolates were typed as specified above except fivefold more concentrated, and adjusted to approximate inhibitor activity as specified above except for the first 7 sera) by using pairs of 96-well plates. Isolate pool control (Pa and Pb) was the coat antigen in the first column of each plate pair. Serum pool control (SP) was placed in the first row of each plate pair. A total of four plate pairs, each accommodating 7 sera and the pool, were required to complete the test panel of 28 patient sera (plate pairs accommodating sera 8 through 28 not shown).

### CELISA

#### Serum matrix

The basic method of measuring antigen-specific antibody binding used in the checkerboard assay was a modification of the original ELISA method of Engvall and Perlmann (2) which was adapted for use with ultrasonically disrupted bacteria immobilized in microtest plate wells as described previously (11). The assay protocol required only the use of commercially available laboratory equipment, such as plastic wash bottles filled with appropriate buffers and manual 8-channel or 12-channel pipetters for use in transferring reagents to standard 96-well MicroTest plates. CELISA absorbance readings were done with a standard MicroTest plate photometer equipped with a serial interface for exporting data to a desktop computer.

Isolates were thawed, ultrasonically disrupted in buffer containing protease inhibitors as specified above except fivemore concentrated, and adjusted to a protein concentration of 5.0 μg/ml (Bradford assay; BioRad, Inc., Richmond, Calif.) in 100 mM bicarbonate buffer at pH 9.8. A multi-isolate test grid was prepared by adding 100 μl of the disrupted isolate suspensions to wells of pairs of test plates (EIA II; ICN, Inc., Irvine, Calif.) in the exact order specified in Table 1. Adsorption of antigens to well surfaces was allowed to take place at ambient temperature for 4 h. Wells were filled with blocking buffer [10 mM N-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid, 0.85% (wt/vol) NaCl, 1% (wt/vol) nonfat powdered milk, 0.1% (vol/vol) Tween 20], incubated for 30 min, and washed three times with the same buffer immediately prior to the introduction of test sera. To find an optimal single serum dilution for use in the CELISA and to confirm the functional quality of the assay reagents, the sera were prescreened at different serial dilutions by conventional ELISA (10) in which the solid phase antigen was a pool of nine clinical isolates and ATCC type strain 43057 (data not shown). A dilution of 1:400 was found to be optimal (see Results).

### Test sera

Test sera were diluted in advance and stored in blocked blank test plate wells arranged in the specified order in which they were to be introduced to isolate-coated wells (Table 1). Sets of 12 diluted sera were then rapidly transferred to isolate coated wells in corresponding rows across the plate pairs with a 12-channel pipetter to minimize incubation timing errors which could result from transferring samples singly. As shown in Table 1, the arrangement of plate wells for testing 22 isolates against 28 sera (with appropriate control pools) required an assay grid consisting of 24 columns by 32 rows of wells established with four pairs of 96-well plates. Plates in the grid were internal controls in the assay. Grid were placed in a cold storage chest overnight to proceed for 30 min, and the reaction was stopped by adding 25 μl of 0.2 N H2SO4 to all wells. The absorbance of the wells at a wavelength of 405 nm was measured with a TiterTek Multiskan MC microtiter plate photometer. The data were exported to computer disk files for statistical analysis.

### Data analysis

The organization of the CELISA data matrix was exactly the same as that of the assay grid described above and shown in Table 1. Each well in the grid (except for the controls) represented a unique combination of one isolate and one serum. After the absorbance was measured for each well in the grid as described above, the resulting absorbance values were placed in a data matrix in which the columns and rows of values indicating isolate IgG binding for different combinations of sera and isolates corresponded exactly to the CELISA grid organization (isolates in columns and sera in rows). For the purposes of this study, the IgG binding profile was defined as the pattern of absorbance values from left to right from one isolate to another in a given serum row in the data matrix, or alternatively, as the pattern of values from one serum to another down a given column corresponding to a single isolate in the matrix. Graphical representations of the data were shown in Fig. 3 and 5.

In order to determine if the isolates could be serologically grouped based on their IgG binding profiles, the CELISA absorbance matrix was analyzed by correlation and hierarchical cluster analysis with SPSS version 7.5 software. The analysis was done in two different ways: (i) with data for all sera included in the matrix (not shown) and (ii) with sera with absorbance values against the isolate pool control values below the median (n = 14) excluded (data shown in tables and figures). Spearman correlations between IgG binding profiles across different rows and down columns were calculated as Pearson’s correlation coefficients for all possible serum and isolate pairs in order to reveal levels of similarity and dissimilarity between different sera.
TABLE 2. Spearman rank correlation between IgG binding profiles of 21 clinical isolates of B. forsythus (designated A through C and E through V) and ATCC 43037 (designated D) tested against 14 patient sera with IgG titers to pooled isolates above the median*

| Isolate | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | T | U | V |
|---------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A       | 1.00 |
| B       | 0.98 | 1.00 |
| C       | 0.26 | 0.67 | 1.00 |
| D       | 0.91 | 0.30 | 0.90 | 1.00 |
| E       | 0.67 | 0.63 | 0.74 | 0.27 | 1.00 |
| F       | 0.63 | 0.62 | 0.64 | 0.66 | 0.56 | 0.79 | 1.00 |
| G       | 0.66 | 0.42 | 0.65 | 0.61 | 0.74 | 0.23 | 0.58 | 0.70 | 0.93 | 1.00 |
| H       | 0.07 | 0.36 | 0.05 | 0.63 | 0.57 | 0.34 | 0.55 | 0.09 | 0.47 | 0.64 | 0.88 | 0.73 | 0.58 | 1.00 |
| I       | 0.65 | 0.52 | 0.64 | 0.74 | 0.73 | 0.33 | 0.59 | 0.79 | 0.93 | 0.93 | 0.52 | 0.84 | 0.90 | 1.00 |
| J       | 0.71 | 0.43 | 0.68 | 0.59 | 0.67 | 0.12 | 0.60 | 0.78 | 0.94 | 0.84 | 0.48 | 0.93 | 0.90 | 1.00 |
| K       | 0.72 | 0.38 | 0.69 | 0.50 | 0.67 | 0.07 | 0.57 | 0.75 | 0.91 | 0.81 | 0.43 | 0.93 | 0.89 | 0.97 | 1.00 |
| L       | 0.62 | 0.23 | 0.85 | 0.42 | 0.77 | 0.57 | 0.90 | 0.58 | 0.49 | 0.50 | 0.23 | 0.49 | 0.54 | 0.56 | 0.48 | 1.00 |
| M       | 0.55 | 0.56 | 0.53 | 0.90 | 0.68 | 0.55 | 0.55 | 0.82 | 0.78 | 0.77 | 0.61 | 0.69 | 0.87 | 0.79 | 0.72 | 0.60 | 1.00 |
| N       | 0.67 | 0.43 | 0.66 | 0.65 | 0.78 | 0.26 | 0.72 | 0.92 | 0.81 | 0.76 | 0.47 | 0.74 | 0.88 | 0.85 | 0.81 | 0.64 | 0.86 | 1.00 |
| O       | 0.68 | 0.50 | 0.65 | 0.68 | 0.76 | 0.27 | 0.67 | 0.86 | 0.83 | 0.79 | 0.56 | 0.74 | 0.84 | 0.89 | 0.81 | 0.64 | 0.89 | 0.92 | 1.00 |
| P       | 0.58 | 0.65 | 0.45 | 0.78 | 0.62 | 0.56 | 0.42 | 0.60 | 0.71 | 0.82 | 0.39 | 0.50 | 0.82 | 0.66 | 0.63 | 0.45 | 0.85 | 0.66 | 0.70 | 1.00 |
| Q       | 0.63 | 0.47 | 0.61 | 0.59 | 0.66 | 0.17 | 0.64 | 0.87 | 0.76 | 0.62 | 0.55 | 0.78 | 0.74 | 0.86 | 0.79 | 0.62 | 0.81 | 0.90 | 0.94 | 0.50 | 1.00 |
| R       | 0.51 | 0.59 | 0.49 | 0.83 | 0.68 | 0.54 | 0.52 | 0.74 | 0.74 | 0.76 | 0.00 | 0.58 | 0.79 | 0.73 | 0.63 | 0.56 | 0.93 | 0.79 | 0.89 | 0.87 | 0.76 | 1.00 |

* Boldface underlined and underlined r values are significant at P < 0.01 and P < 0.05 levels, respectively. Isolate IgG binding profile in this analysis is defined as the pattern of CELISA absorbance values from one serum to another down a given column in the CELISA data matrix (see Materials and Methods). Sera with absorbance values below the median for wells coated with the pooled isolate control were excluded from the correlation analysis (see Results).

and isolates (Tables 2 and 3). The correlations between profiles of the pooled serum and isolate controls in the grid were also calculated to identify correlation coefficients to be expected when isolates or sera were almost identical in their IgG binding profiles. In addition, the binding profiles of all serum and isolate pairs were analyzed by using hierarchical clustering algorithms. More specifically, we used average linkage between-groups method and Spearman correlation coefficients as a similarity measure for IgG binding profiles of different isolates across different sera and for different sera across different isolates. Hierarchical graphically outlining cluster analysis results were generated by using options available in the above-mentioned statistical package and were redrawn to improve their graphical quality.

Statistical significance between the median absorbance values corresponding to different groups of isolates or sera were determined by using the Wilcoxon signed rank test.

TABLE 3. Spearman rank correlation between IgG binding profiles of sera from 14 patients with IgG titers above the median tested against 22 isolates of B. forsythus*

| Sera | Correlation coefficient |
|------|-------------------------|
| 1    | 1.00                    |
| 4    | 0.95                    |
| 8    | -0.46                   |
| 11   | 0.34                    |
| 14   | 0.48                    |
| 15   | 0.55                    |
| 16   | 0.06                    |
| 17   | 0.17                    |
| 18   | 0.50                    |
| 20   | 0.35                    |
| 25   | 0.39                    |
| 26   | 0.42                    |
| 27   | -0.05                   |
| 28   | -0.04                   |

* Boldface underlined and underlined r values are significant at P < 0.01 and P < 0.05 levels, respectively. Isolate IgG binding profile in this analysis is defined as the pattern of CELISA absorbance values from one isolate to another down a given row in the CELISA data matrix (see Materials and Methods). Sera with absorbance values below the median for wells coated with the pooled isolate control were excluded from the correlation analysis (see Results).

RESULTS

Based on the results of a conventional ELISA in which the solid phase antigen was a pool of nine clinical isolates and ATCC 43037 and the sera were serially diluted, a dilution of 1:400 was found to be optimal for use in the CELISA. Absorbance values at a 1:400 serum dilution correlated well with IgG titers (ELISA units) against the isolates measured relative to a dilution curve standard generated using a pool of patient sera (10). IgG binding (absorbance) at this dilution was within the log linear region of the dilution curves of all positive sera. Thus, reasonable accuracy was expected in defining IgG bind-
ing profiles of individual isolates and sera in the CELISA. ELISA unit values in the conventional ELISA indicated that a wide range of IgG titers against pooled isolates existed across different patient sera (data not shown).

The mean absorbance values for isolate pool controls in the CELISA were in general agreement with conventional ELISA IgG titers (Fig. 1). Values for different sera varied from over 2.7 down to 0.1, just above the absorbance background value measured in wells without serum (approximately 0.05 absorbance units). The median absorbance for sera in wells coated with the isolate pool (0.79) fell between the values corresponding to serum 14 and serum 10 (Fig. 1). This median absorbance value was used to select absorbance values corresponding to positive sera for use in the correlation analyses (Tables 2 and 3) and the cluster analysis of the isolates (see Fig. 4). Sera with values below the median were excluded from the analyses to reduce the influence of background absorbance noise in the CELISA. Absorbance values for the isolate pool control wells on different plates pairs in the assay (Pa and Pb; Table 1) in the CELISA were in close agreement (mean difference, less than 6.5%) for sera above the median. This indicated that absorbance data rescaling to correct for incubation timing error between plate pairs was not necessary. As shown in Fig. 2, the isolate pool values (Pa and Pb) for the highest serum, lowest serum, and median were in good agreement. Pa and Pb medians were not significantly different as determined by the Wilcoxon signed rank test. However, the medians of the sera tested against several individual isolates (e.g., D, H, U, and V) were significantly lower than corresponding isolate pool values ($P < 0.001$, Wilcoxon signed rank). Other isolates (e.g., A, N, and I) gave median serum values not statistically different from those of the isolate pool controls. Figure 2 also shows that all isolates were positive for IgG binding in at least one serum in the test panel, with absorbance values greater than 1.5 in all cases, and that all isolates were negative for at least one serum in the set.

Although finding good agreement between the magnitudes of corresponding isolate pool values on different plates demonstrated good assay accuracy, the primary use of the isolate pool repeat columns in the CELISA was to provide a negative control for the assessment of antigen variation between different individual isolates. As can be seen in Fig. 3, the isolate pool control IgG binding profiles were very similar, as would be expected if two isolates with identical antigens were being compared. The Spearman correlation coefficient between the isolate pool repeat columns across all 28 sera and across only those above the median was 0.97 ($P < 0.01$) in both cases. In sharp contrast, the profiles of individual isolates were quite dissimilar in some cases, as indicated by low correlation coefficients (e.g., F and U, $r = 0.17$ [Table 2 and Fig. 3], and F and L, $r = 0.09$ [Table 2]), and were moderately dissimilar in other cases, as indicated by intermediate $r$ values. In some cases, however, different isolates appeared to be antigenically identical (e.g., A and C [$r = 0.98$] and B and K [$r = 0.96$]). Some sera exhibited absorbance values near background in all isolates (e.g., sera 5, 9, and 13) and did not appear to discriminate well between different isolates, while others gave absorbance values greater than twofold higher than that of the pooled isolate median (e.g., sera 1, 4, and 8) and appeared to discriminate greatly between different isolates, such as F and U (Fig. 3).

To systematically reveal all of the possible serogroups represented in this particular collection of isolates, we subjected their IgG binding profiles across the 14 sera with titers to pooled isolates above the median to hierarchical cluster analysis. The results are presented as a dendrogram in Fig. 4. The isolates sorted into two main clusters, A through D and B through F. The first cluster contained most of the isolates and broke down into three well-defined subclusters, A through P, H through L, and Q through D. The second main cluster contained one well-defined subcluster, B through K, and a single isolate, F, that did not cluster with any of the other isolates.

A function analogous to that of the isolate pool control wells was served by the pooled serum control wells included in the test panel. The median serum values not statistically different from those above the median was 0.97 ($P < 0.01$) in both cases. In sharp contrast, the profiles of individual isolates were quite dissimilar in some cases, as indicated by low correlation coefficients (e.g., F and U, $r = 0.17$ [Table 2 and Fig. 3], and F and L, $r = 0.09$ [Table 2]), and were moderately dissimilar in other cases, as indicated by intermediate $r$ values. In some cases, however, different isolates appeared to be antigenically identical (e.g., A and C [$r = 0.98$] and B and K [$r = 0.96$]). Some sera exhibited absorbance values near background in all isolates (e.g., sera 5, 9, and 13) and did not appear to discriminate well between different isolates, while others gave absorbance values greater than twofold higher than that of the pooled isolate median (e.g., sera 1, 4, and 8) and appeared to discriminate greatly between different isolates, such as F and U (Fig. 3).

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CELISA design (Table 1). These controls permitted interplate assay bias to be monitored and provided a standard against which absorbance values could be rescaled to correct the assay bias, if necessary. However, their primary purpose was to provide a negative control for the assessment of variation in isolate recognition profiles between different sera. As shown in Fig. 5, repeat rows of wells containing the serum pool revealed very similar IgG binding profiles across the 22 B. forsythus isolates. The Spearman rank correlation between serum pool controls was positive and significant ($r = 0.97, P < 0.01$). In sharp contrast, the profiles for different individual sera were extremely dissimilar in some cases (e.g., sera 1 and 8, $r = -0.46, P < 0.05$ [Fig. 5 and Table 3]), while the profiles for other serum pairs were almost identical (e.g., sera 1 and 4, $r = 0.95, P < 0.01$ and sera 11 and 26, $r = 0.90, P < 0.01$). Cluster analysis of the data for sera which had pooled isolate IgG titers above the median ($n = 14$) revealed two main serum clusters. The first cluster contained three subclusters, sera 1 and 4, sera 17, 25, 11, 26, and 14, and sera 18, 20, and 15. The second main cluster contained four tightly clustered sera, 16, 28, 8, and 27. The highest degree of concordance between correlation coefficients (Table 3) was between those of sera 1 and 4, indicating that they were the most closely related sera in the collection (dendrogram not shown). Furthermore, sera 8 and 28, the sera with the highest pooled isolate titer ranks (Fig. 3), had profiles very different from those of sera 1 and 4, which ranked third and fourth highest in titer, as indicated by their cluster membership and the correlation analysis (Table 3).

The serum pool controls occupied the first column on each plate and the isolate pool occupied the first row (Table 1). Consequently, the isolate pool was assayed on each plate against the serum pool, thus providing master pool/pool control absorbance values which could be used to rescale the CELISA absorbance data, if necessary, to correct for interplate assay bias that could result from incubation timing errors and other factors which might systematically affect data from different plates. The single-well pool/pool control data were in good agreement with mean differences observed between plate pairs in the assay grid (less than 6.5%) so data rescaling was not considered necessary. However, if larger numbers of plates were to be processed, data rescaling could become necessary.
The potential for rescaling the absorbance data from different plates based on the included controls makes it possible to expand the number of isolates and serum combinations analyzed in the CELISA without the necessity of processing all plates within the same assay run.

**DISCUSSION**

*B. forsythus* is one of the well-documented etiological agents of destructive periodontal diseases (1), but information concerning the role of humoral and local immunity to this species in periodontal infections is currently very limited. One of the fundamental aspects of the immune response to *B. forsythus* that needs to be elucidated is whether or not the species exists in multiple serotypes as recognized by patients. Recently, a study revealed that *B. forsythus*, like *P. gingivalis* and other oral species, exists in multiple clonal types as measured by restriction endonuclease ribotyping (12). Learning whether the observed genetic diversity of *B. forsythus* is accompanied by significant serological variation in antigen expression among clinical isolates is central to understanding the role of humoral immunity to the species in periodontitis.

The novel CELISA system applied in the present study is an efficient method of detecting variation in IgG binding profiles of individual *B. forsythus* isolates across different patient sera. In addition, the assay system reveals heterogeneity in the serum IgG responses of different patients to isolates within different serological clusters. Unlike ELISA versions designed to find antigen-specific antibody concentrations relative to a dilution curve of a standard serum, the CELISA required the use of just one standard optimal dilution of all test sera. It should be emphasized that an optimal serum dilution must be determined in advance for a given set of sera and coating antigens to avoid errors due to plateau effects caused by specific antibody concentration being too high relative to the amount of antigen available on the solid phase. Otherwise, the standard serum concentration should be high enough that specific antibody binding can be detected well above the background absorbance noise of the assay.

A key feature of the CELISA design was the inclusion of two different controls: a pooled isolate control and a pooled serum control. These controls provided both a means of monitoring assay bias and assessing assay accuracy. In addition, and of primary importance, they also provided negative controls against which to judge variation in IgG binding profiles from one serum to another and from one isolate to another. In the present application of the CELISA system, the control data revealed a high degree of interplate reproducibility and overall assay accuracy. Consequently, unequivocal evidence indicating that *B. forsythus* clinical isolates can be reliably sorted into different serological groups as recognized by serum IgG of patients with rapidly progressive periodontitis was obtained. Highly significant positive correlation (*r* = 0.97, *P* < 0.01) was observed for repeats of the isolate pool control columns of the test grid as shown in Fig. 5. This level of correlation between isolate pairs was therefore taken as an indication that their antigen composition was virtually identical, while negative or near-zero correlation between pairs was considered evidence that the isolates were members of different serological clusters. Hierarchical cluster analysis showed that the collection of 22 isolates could be sorted into at least four serological subclusters, as shown by the dendrogram plot in Fig. 4, suggesting that the patients in the study had been infected with and mounted an IgG response to different *B. forsythus* serotypes.

The isolate pool control data served another important purpose in the data analysis: data for sera with low or negative
antibody levels could be excluded from the analysis to minimize the confounding effects of random variation in the background absorbance noise on the clustering and correlation results. The analyses shown in the correlation tables and the dendrogram were in fact done with data from sera with pooled isolate absorbance values above the median for all 28 sera. Excluding data for weakly positive and negative sera resulted in tighter clustering of isolates into different serological groups.

In an analogous way, the pooled serum control row repeats served as a negative control for assessing heterogeneity in the recognition of isolates by different pairs of sera. The observation that the serum pool row repeats and some pairs of sera correlated positively and significantly while other pairs of sera exhibited negative or near-zero correlations strongly suggests that different patients may be infected with and respond to different serotypes of \textit{B. forsythus}. Consequently, it follows that some of the potentially useful immunogenic components of the species which might be good vaccine candidates need to be assessed for cross-reactivity across the full range of serological variants of the species if the goal is to achieve broad-spectrum protection.

Data rescaling was not necessary in the present application because of the observed close agreement in comparable repeated absorbance measurements of pooled isolate and serum controls rows on different assay plates, although rescaling would be possible and advantageous in applications with larger numbers of test plates. Another advantageous feature of the new CELISA design was that hierarchical clustering of isolates was based on the Spearman rank correlation which was completely unaffected by systematic variation in the data measurement scale. Because it was the correlation in absorbance profiles that defined clustering, factors which uniformly affect all absorbance values within a given row or plate pair in the test grid did not bias the results. To simulate systematic error that might result from letting one plate pair incubate with substrate longer than another, we read the same pair of plates repeatedly at 5-min time intervals after the introduction of phosphatase substrate. Absorbance values increased between plate readings in accordance with linear enzyme-substrate reaction kinetics, but the correlation between absorbance profiles across the same wells at different development times was greater than 0.98 ($P < 0.01$) for all plate pair rows (data not shown).

These results strongly suggest that different patients with rapidly progressive periodontitis mount a humoral immune response to different serological variants of \textit{B. forsythus}. However, whether the presence of specific IgG in positive sera is indicative of infection of individuals with different serotypes of the organism needs further clarification, in part, because the isolates tested here were heterologous relative to the sera tested in the CELISA. Nonetheless, the results provide valuable insight into the nature of the antigens of \textit{B. forsythus} recognized by serum antibodies of patients and suggest that additional work should be undertaken to more precisely determine exactly how many clinically relevant serotypes of this species play a role in different types of periodontitis and whether the infection of a given individual may involve more than one serotype.

In addition to being the first report of the serological heterogeneity of \textit{B. forsythus} isolates and one of the few which have attempted to elucidate the role of humoral immunity to the species in human periodontal diseases, this study has demonstrated the efficacy of a novel and generally applicable method of serologically classifying clinical isolates of pathogenic bacteria.

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