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Fluorescence Immunoassay for Detecting Periodontal Bacterial Pathogens in Plaque

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A particle concentration fluorescence immunoassay has been modified into a bacterial concentration fluorescence immunoassay (BCFIA) to rapidly detect periodontopathic bacteria in human plaque samples. The BCFIA utilizes fluorescently tagged monoclonal antibodies (MAbs) directed against the lipopolysaccharide of selected gram-negative plaque bacteria. Microorganisms closely associated with periodontal disease that can be identified in plaque with the BCFIA include *Porphyromonas gingivalis*, *Bacteroides intermedius*, *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Eikenella corrodens*. Briefly, the procedure involved mixing a patient's plaque sample or other bacterial preparation with a species-specific fluorescein isothiocyanate-labeled MAb in a specialized microtiter plate. This mixture was incubated to allow binding of the MAb to its homologous bacteria. The bound and unbound fluorescent tagged MAbs were separated by filtration in the modified microtiter plate, and the total bacterial bound fluorescence was determined with a fluorimeter. The number of a specific bacterial species in a given plaque sample or other bacterial suspension was estimated by reference to a primary standard carried through the BCFIA. The lower detection limit of the BCFIA was 10^3 to 10^4 bacterial cells from single cultures of bacteria or 10^4 bacterial cells in mixed cultures. The coefficient of variation within and between plates for each of the five bacterium-specific MAbs in screening plaque for the periodontal pathogens was <10%. These results demonstrate that microbes in plaque can be used as the solid phase in the BCFIA to detect and quantitate MAbs associated with specific bacteria quickly and reliably.

Human periodontal disease is associated with a complex microflora. Over 300 bacterial species may be cultured from the periodontal pockets of different individuals, and as many as 30 to 50 bacterial species may be recovered from a single diseased site. Relatively few of these bacterial species, however, are likely responsible for the transition from health to periodontal disease. The proportion of gram-negative, anaerobic bacteria has been shown to increase in human subgingival plaque as the severity of periodontal disease increases (21, 25). Included among these gram-negative anaerobic bacteria are *Porphyromonas gingivalis* (formerly *Bacteroides gingivalis*), *Bacteroides intermedius*, *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens*, and *Fusobacterium nucleatum*. Previous studies have associated these microorganisms with various degrees of inflammation and a variety of periodontal disease conditions, using cultivable flora approaches (6, 12, 14, 17, 22, 24). Cultivable flora techniques for the analysis of plaque, however, are time-consuming and expensive. It may take 2 to 4 weeks to identify gram-negative bacteria found in a particular plaque sample. Therefore, as part of on-going, large-population, longitudinal studies to determine bacterial risk factors for the development and progression of periodontal diseases, we sought alternatives to cultivation-based bacterial identification.

A particle concentration fluorescence immunoassay (PCFIA) which utilized polystyrene beads coated with immunoglobulin as the solid phase was developed by Jolley et al. (13). The PCFIA was reported to be as or more sensitive and could more quickly evaluate samples than the enzyme-linked immunosorbent assay (ELISA). A variety of investigators have used the PCFIA or a modification of the technique to quantitate immunoglobulins or other molecules from various sources (3, 5, 15, 18). The use of bacteria as the solid phase, instead of polystyrene beads, in a modification of the PCFIA for analysis of antibodies to surface bacterial antigens has also been described recently by Schwan et al. (19), who described the technique as the bacterial concentration fluorescence immunoassay (BCFIA). The purpose of this investigation was to modify and utilize the BCFIA for the rapid and reliable identification and quantitation of periodontopathic bacteria in human plaque specimens. The BCFIA technique utilizes bacterial lipopolysaccharide (LPS)-specific monoclonal antibodies (MAbs). Bacteria are the solid phase to which the LPS-specific fluorescein isothiocyanate (FITC)-tagged MAbs are directed. The bound and unbound fluorescent tagged MAbs are separated by filtration in a modified microtiter plate, and the total bacterial bound fluorescence is determined with a fluorimeter. The BCFIA, which takes advantage of species-specific MAbs directed against plaque pathogens, could have widespread significant research, diagnostic, and therapeutic applications in infectious diseases (26), including periodontal diseases.

MATERIALS AND METHODS

Species-specific LPS MAbs. Bacterial species-specific MAbs (3M Corporation, St. Paul, Minn.) were prepared by procedures described previously (9). Briefly, MAbs secreted by hybridomas constructed from splenic leukocyte fusions with NS/I mouse myeloma cells were screened by the ELISA against purified LPS preparations (4, 23) from *P. gingivalis*, *B. intermedius*, *A. actinomycetemcomitans*, *F.*

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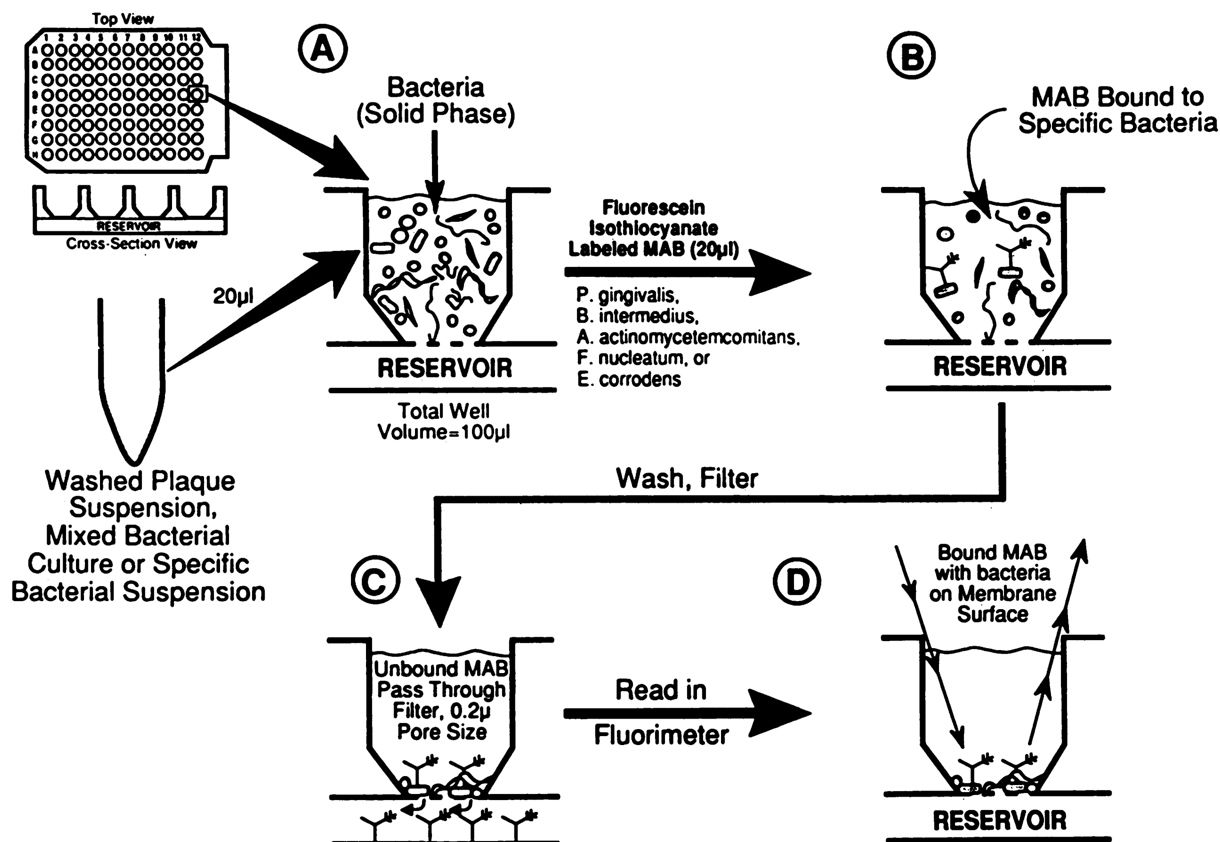

FIG. 1. BCFIA procedure for evaluating plaque samples, mixed bacterial cultures, or a specific bacterial suspension.

nucleatum, and *E. corrodens*. Heterologous cross-reactions with 41 oral bacterial type strains, both gram-positive and gram-negative, obtained from the American Type Culture Collection were <5% of the intensity of the homologous reaction for each of the five bacterium-specific MAbs (19a).

BCFIA. The general principle of the PCFIA has been described in an earlier report (13). The modified BCFIA is outlined in Fig. 1 in relation to evaluating plaque samples or mixed bacterial cultures for specific bacteria or identifying bacteria in a single bacterial suspension. The BCFIA was performed in a specially designed 96-well microtiter plate (Fluoricon Assay Plate; Baxter HealthCare Corp., Pandex Div., Mundelein, Ill.). Whole bacterial cells served as the solid phase in the BCFIA procedure. Prior to carrying each plaque sample or bacterial preparation through the immunoassay, cells were washed twice in distilled water–0.05% Tween and then resuspended in 500 µl of phosphate-buffered saline (PBS)–0.05% Tween plus 1% goat serum. Twenty microliters of a bacterial or plaque cell suspension containing $\leq 5 \times 10^7$ total bacteria was placed in each of two wells of the microtiter plate. To one well was added 20 µl of a bacterial species-specific, FITC-labeled (Isomer I; Research Organics, Cleveland, Ohio) MAb, and the plate was incubated in the dark at 22°C for 20 min and then washed twice with PBS plus 0.0125% Nonidet P-40 and filtered under 25-mm vacuum. The other well containing the bacterial or plaque suspension served as a control and was treated with FITC-labeled MOPC, a myeloma protein at a concentration of 5 µg/ml and a fluorescein/protein ratio of 10, and assayed in parallel to the test mixture. Species-specific MAbs utilized in the immunoassay included those against *P. gingivalis*, *B. intermedius*, *A. actinomycetemcomitans*, *F. nucleatum*, or *E. corrodens*. The pore size of the membrane filter was 0.2 µm, allowing bacteria with or without tagged MAb to be trapped on the membrane surface while the unbound MAb passed through the filter with the buffer wash. Total bacterial bound fluorescence (excitation, 485 nm) was determined in a fluorimeter (Pandex Fluorescence Concentration Analyzer; Baxter HealthCare Corp.) at a wavelength of 535 nm. Total bound bacterium-specific fluorescence was obtained by subtracting nonspecific fluorescent binding in the control well from the total fluorescent-antibody binding in the test well. The bacterial cell equivalents were determined by comparison to a standard curve.

BCFIA evaluation with single cultures of periodontopathic bacteria. The sensitivity of the immunoassay for screening single cultures of *P. gingivalis*, *B. intermedius*, *F. nucleatum*, *E. corrodens*, or *A. actinomycetemcomitans* was determined by the BCFIA as described above. Twenty microliters of 10^2 to 10^6 washed log-phase bacterial cell preparations was added to wells of the immunoassay plate followed by one of the FITC-labeled, species-specific MAbs. The MAb concentration and fluorescein/protein ratio, respectively, for each species-specific MAb preparation used in these experiments were as follows: *P. gingivalis*, 2.6 µg/ml, 4.6; *F. nucleatum*, 3.7 µg/ml, 4.2; *A. actinomycetemcomitans*, 4.3 µg/ml, 4.5; *E. corrodens*, 5.2 µg/ml, 6.3; *B. intermedius*, 7.1 µg/ml, 11.1.

BCFIA evaluation with periodontopathic bacteria in a mixed culture. In an effort to determine whether a mixed culture of bacteria would have an effect on the ability of the

TABLE 1. Preparation of dilution buffers for use in the BCFIA[a] for evaluation of periodontopathic bacteria in a mixed culture

Bacteria	Concn (bacteria per ml) in dilution buffer[b]:				
	A	B	C	D	E
P. gingivalis	0	10^7	10^7	10^7	10^7
B. intermedius	10^7	0	10^7	10^7	10^7
F. nucleatum	10^7	10^7	0	10^7	10^7
A. actinomycetemcomitans	10^7	10^7	10^7	0	10^7
E. corrodens	10^7	10^7	10^7	10^7	0
S. mutans	3.5×10^7	3.5×10^7	3.5×10^7	3.5×10^7	3.5×10^7

[a] After dilution, buffers were spiked with 2.5×10^7 bacteria per ml, as follows: buffer A, *P. gingivalis*; B, *B. intermedius*; C, *F. nucleatum*; D, *A. actinomycetemcomitans*; E, *E. corrodens*. The final total concentration of bacteria in each buffer with the spiked bacteria was 10^8/ml. See Materials and Methods.
[b] Bacteria were suspended in PBS, with the pH adjusted to 7.4.

BCFIA to detect a specific periodontopathic bacterium, competition experiments were performed (Table 1). Dilution buffers A, B, C, D, and E were prepared by adding log-phase cultures of the gram-negative bacteria *P. gingivalis*, *B. intermedius*, *F. nucleatum*, *A. actinomycetemcomitans*, and *E. corrodens* and/or the gram-positive bacterium *Streptococcus mutans* to PBS, pH 7.4, at the concentrations given in Table 1. A portion of buffer A, B, C, D, or E was then spiked with 2.5×10^7 *P. gingivalis*, *B. intermedius*, *F. nucleatum*, *A. actinomycetemcomitans*, or *E. corrodens* per ml, respectively. The final total concentration of bacteria in each buffer with the spiked bacteria was 10^8/ml. This suspension of bacteria was then diluted in buffer A, B, C, D, or E (without the spiked bacteria) and titrated against species-specific MAbs at a concentration of 1 μg/ml, using the BCFIA. With this dilution scheme, the concentration of the spiked bacteria decreased while the concentration of the other bacteria remained the same. The fluorescein/protein ratios for the MAb preparations used in these experiments were as follows: *P. gingivalis*, 5.6; *F. nucleatum*, 6.2; *A. actinomycetemcomitans*, 4.2; *E. corrodens*, 4.7; *B. intermedius*, 7.1.

Primary standard for BCFIA. The standard curve was established by examining the relationship between fluorescence and bacterial numbers in the BCFIA. The least-squares method was used to determine the best-fitting mathematical model. Preparation of the standard curve utilized LPS-enriched coated polystyrene particles as the solid phase in the BCFIA. For each of the bacteria, *P. gingivalis*, *B. intermedius*, *A. actinomycetemcomitans*, *F. nucleatum*, and *E. corrodens*, 200-ml log-phase cultures were washed three times in Dulbecco's PBS buffer and resuspended in 20 ml of the same buffer, pH 7.4. The cells were sonicated (Branson Sonifier, model 350; Branson Sonic Power Co., Danburg, Conn.) for three 1-min bursts at an output of 10% and centrifuged for 15 min at 12,000 × *g* to remove unbroken cells. The supernatant from each of the bacterial preparations was combined with 2.0 ml of 0.85-μm fluoricon polystyrene assay particles (Baxter HealthCare Corp.), and the combination was rotated at 0.5 rpm for 12 to 18 h at 22°C. The beads were then washed in PBS plus 0.0125% Nonidet P-40 and resuspended in 40 ml of the same buffer with 0.5% sodium azide. The LPS-coated beads were then standardized by immunoassay to suspensions of whole bacterial cells and assayed with their homologous MAbs, using the same procedure as described above in the BCFIA. Simultaneously, suspensions of each of the five bacteria from log-phase cultures were adjusted, using a Petroff-Hausser chamber, at concentrations ranging from 1.0×10^3 to 1.0×10^6 bacteria per well and then immunoassayed. Each preparation of antigen-coated beads was then diluted with bovine serum albumin plus 1% goat serum-coated beads to give a standard corresponding to a specific number of bacteria in the BCFIA. Specific bead preparations (*P. gingivalis*, *B. intermedius*, *A. actinomycetemcomitans*, *F. nucleatum*, or *E. corrodens*) ranged in concentration from 0.250 to 0.016% (milligrams of beads/volume of buffer) and were used to prepare the standard curve. The correlation coefficient between bacterial numbers and bead concentration was consistently ≥0.84 for all species. Calibrated bead suspensions were stored at 4°C until used in the BCFIA. Each microtiter plate which contained plaque or other bacterial samples also contained primary standards prepared in duplicate. The average of the two primary standards for six or more different bead concentrations was used to prepare the standard curve and determine the number of bacteria in test samples.

BCFIA reproducibility study. Variability for the BCFIA determinations among wells and among plates was assessed by repeatedly assaying selected pooled plaque samples in the BCFIA (Fig. 2). Precision of the immunoassay was established by determining the intra- and interassay coefficients of variation.

RESULTS

BCFIA for detection of pure cultures of periodontopathic bacteria. Titration of the MAbs against their homologous bacteria by the BCFIA can be seen in Fig. 3. The lower detection limit of the immunoassay for detecting pure cul-

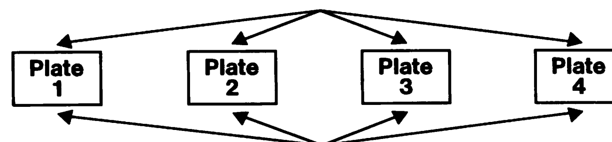

FIG. 2. Protocol for determining precision (inter- and intraassay variability) of the BCFIA.

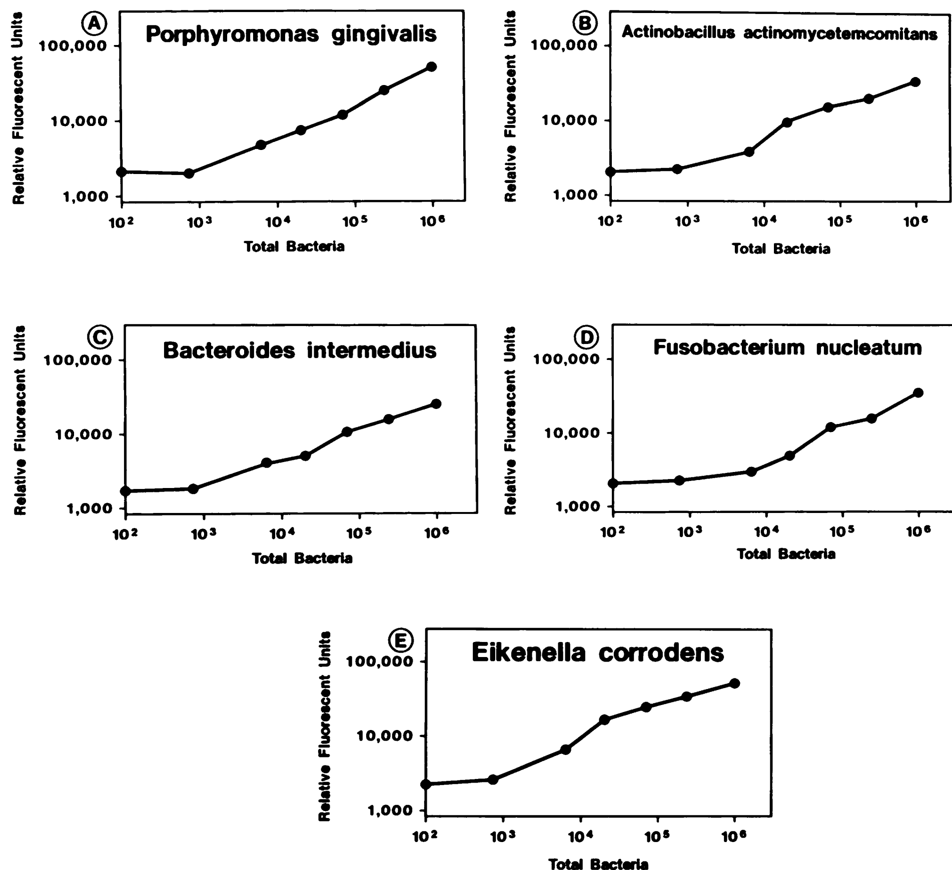

FIG. 3. Titration of species-specific MAbs against their homologous bacteria, using the BCFIA.

tures of *P. gingivalis*, *B. intermedius*, *F. nucleatum*, *E. corrodens*, and *A. actinomycetemcomitans* was $10^{3.5}$ to 10^4 bacterial cells. In addition, there was an approximately linear response between relative fluorescence units and total bacteria over the range of 10^4 to 10^6 bacterial cells.

BCFIA detection of periodontopathic bacteria in a mixed culture. Through a competition experiment, the effect of a mixed culture of bacteria on the sensitivity of the BCFIA for detecting specific bacteria was investigated (Fig. 4). There was a general linear response between relative fluorescence units and log bacteria. It can be observed that, even in the presence of high levels of other bacteria, the BCFIA (using each of the five species-specific MAbs) has a lower detection limit value of at least 10^4 bacterial cells. An example of the relationship between the number of bacteria and fluorescence units obtained in the BCFIA for mixed (competitive) and single (noncompetitive) bacterial cultures is also shown in Fig. 4. The average increases in relative fluorescence units corresponding to a 100-fold increase in bacteria for each MAb and its homologous bacteria in competition and noncompetition experiments were similar. This observation gave supporting evidence that other bacteria in the mixed culture did not interfere with the bacterium-specific MAb reacting with its homologous bacteria in the BCFIA.

Primary standard in the BCFIA. Several models of least-squares curve fitting were tried, and a linear model of the logarithm of dependent and independent variables provided the best fit. In the BCFIA, the standard curve has the form $\log y = a + b \log x$, where y indicates fluorescence and x indicates bacterial counts. The fluorescence of a plaque sample corrected for background was determined in the BCFIA and compared with the standard curve. By using the standard curve, plaque sample fluorescence units were converted into bacterial counts.

An example of converting fluorescence values obtained for a plaque sample into specific bacterial counts by using the BCFIA is illustrated in Fig. 5. For the standard curve, the relationship $\log y = 1.43 + [0.44 \cdot \log (x)]$ was derived. A plaque sample with a fluorescence of 6,151 and corresponding background fluorescence of 1,382 U was determined in the BCFIA. Therefore, with the above equation:

$$\underset{\text{(test)}}{\log (6{,}151} - \underset{\text{(background)}}{1{,}382)} = 1.43 + [0.44 \cdot \log (x)]$$

or, solving for $\log (x)$

$$\log (x) = [\log (4{,}769) - 1.43]/0.44 = 5.11$$

corresponding to $x = 10^{5.11} = 128{,}825$ or 1.29×10^5 specific bacterial counts in 20 μl of the plaque sample. Twenty microliters of the original 500 μl of the plaque sample was used in the immunoassay. Therefore, the total number of bacterial cells in the plaque sample collected was determined by multiplying 1.29×10^5 specific bacterial counts by 25 (500 μl/20 μl) to give 3.23×10^6 bacterial cells in the total plaque sample collected. Expressed as concentration, this would be 3.23×10^6/500 μl or 6.46×10^6 bacteria per ml.

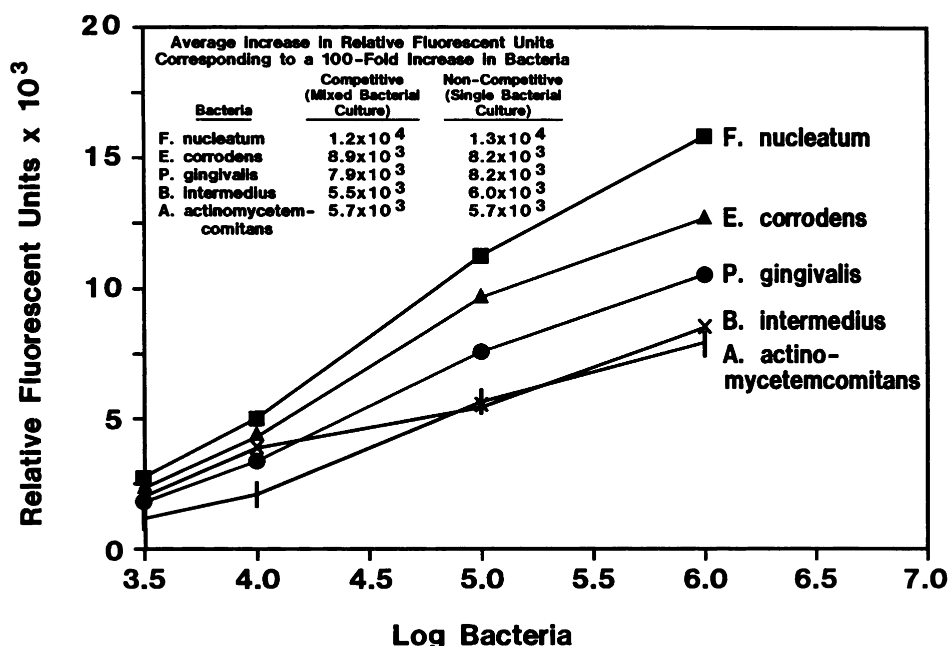

FIG. 4. BCFIA for detection of periodontopathic bacteria in a mixed culture of bacteria.

BCFIA reproducibility study. The interassay precision of the BCFIA, i.e., the plate-to-plate variability, is shown in Table 2. The coefficient of variation for repeated runs of the BCFIA in detecting *A. actinomycetemcomitans*, *B. intermedius*, *P. gingivalis*, *E. corrodens*, and *F. nucleatum* was consistently <10%. The intraassay or well-to-well precision of the immunoassay for separate plates utilizing each of the five species-specific MAbs is shown in Table 3. The coefficient of variation was determined by comparison of wells of single plates when the immunoassay was performed. The coefficient of variation for the intraassay experiments with the BCFIA for the five bacteria was <10% in all cases.

DISCUSSION

A sensitive bacterial solid-phase fluorescence immunoassay was developed with the capability of rapidly screening large numbers of plaque samples for specific plaque bacteria; the BCFIA has distinct advantages over the more traditional cultural methods. With cultivable flora approaches, it may take 2 to 4 weeks to evaluate plaque samples for *P. gingivalis*, *B. intermedius*, *A. actinomycetemcomitans*, *F. nucleatum*, and *E. corrodens*. The BCFIA, on the other hand, can analyze plaque samples for these bacteria in 1 to 2 h. Also, in our laboratory, the time to perform the BCFIA was approximately one-half the time it took to perform the ELISA in evaluating the same samples for specific bacteria. In addition, whereas the ELISA may modify an antigen by binding to a solid surface, the BCFIA offers a significant advantage of presenting a bacterial antigen without binding to a solid surface (19). Cultivable flora methods involve a considerable amount of labor, which is greatly reduced with the BCFIA. Besides having the capacity to screen plaque samples for pathogenic bacteria rapidly, the BCFIA has sensitivity levels equivalent to cultural methods. In our experience, the BCFIA detected between 1,000 and 10,000 cells of the specific pathogenic microorganisms in a mixed bacterial sample. Schwan and co-workers recently reported a solid-phase fluorescence immunoassay that uses bacteria as the solid phase to screen antibodies produced against

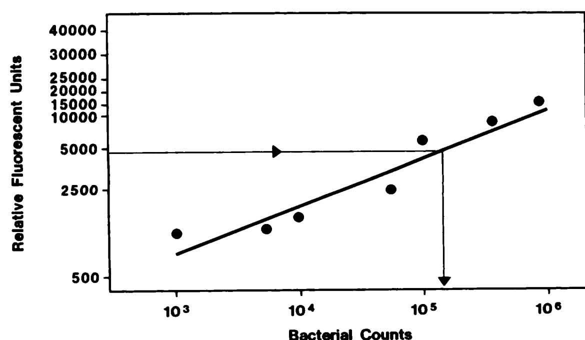

FIG. 5. Illustration of method used to convert fluorescence for a plaque sample determined by the BCFIA into specific bacterial counts. Heavy solid line with circles represents standard curve.

TABLE 2. Interassay precision (plate-to-plate variability), based on coefficient of variation, of the BCFIA in evaluating for specific bacteria in a pooled plaque sample

Bacteria	Mean fluorescence count[a]	SD	% Coefficient of variation
A. actinomycetemcomitans	15,246	922	6.05
B. intermedius	8,951	464	5.19
P. gingivalis	7,134	484	6.79
E. corrodens	16,147	121	7.53
F. nucleatum	13,141	1,242	9.45

[a] Based on taking the average of fluorescence counts determined in 96 wells of a microtiter plate and then determining the mean across four microtiter plates for *A. actinomycetemcomitans*, *B. intermedius*, and *P. gingivalis* and three microtiter plates for *E. corrodens* and *F. nucleatum*.

TABLE 3. Intraassay precision (well-to-well variability), based on coefficient of variation, of the BCFIA in evaluating for specific bacteria in a pooled plaque sample

Expt	Mean % coefficient of variation[a]				
	A. actinomycetemcomitans	*B. intermedius*	*P. gingivalis*	*E. corrodens*	*F. nucleatum*
1	4.70	5.12	8.08	9.50	9.51
2	5.26	5.20	5.50	6.26	4.86
3	5.60	6.20	6.34	6.48	8.37
4	5.80	2.50	5.53	ND	ND

[a] Based on taking the average of fluorescence counts determined in a 96-well microtiter plate. This experiment was repeated four times with *A. actinomycetemcomitans*, *B. intermedius*, and *P. gingivalis* and three times with *E. corrodens* and *F. nucleatum*. ND, Not determined.

surface antigens from a clinical isolate of *Escherichia coli* (19). These investigators, also using bacterium-specific antibodies, demonstrated that the BCFIA was 50-fold more sensitive in bacterial detection than the ELISA and was faster, with uniform reproducibility. Bacterium-specific MAbs, when used in the BCFIA, will detect both viable and nonviable bacterial cells in a plaque sample, whereas cultivable flora techniques only have the capacity to detect viable cells. This is important since LPS, whether from dead cells or associated with live bacterial cells, would be expected to contribute to the inflammatory response and associated destructive processes seen in periodontal disease (8). Also, MAbs used for the detection of specific periodontal bacterial pathogens offer significant advantages over polyclonal antisera due to their restricted specificity (2, 27). Therefore, MAbs in diagnostic tests reduce false-positive results caused by cross-reacting antigens on other microorganisms relative to polyclonal antisera.

There are a number of assay parameters which are crucial for optimal results with the BCFIA. Bacterial concentrations which allowed optimal filtering in the fluoricon microtiter assay plate were $\leq 5 \times 10^7$ bacteria per well. With higher numbers of bacteria, the 0.2-μm-pore-size membrane filter becomes increasingly clogged and impairs passage of fluid, smaller particles, or unbound label through the membrane filter. Since plaque samples may contain as many as 10^9 bacteria, they must be serially diluted prior to evaluation for specific microorganisms by the immunoassay. Also, it was important to add the labeled MAb to a dispersed plaque sample contained within the well. When the labeled MAb was added to the well first followed by a portion of the plaque sample, the specific antibody binding was reduced and background fluorescence increased. The fluorescent signal in the test well was also significantly higher than that of the control well. To adjust for run-to-run differences in the BCFIA, it was necessary to prepare a standard curve on each microtiter plate. The standard curve, which was prepared and run in duplicate on each plate used in the BCFIA, utilized LPS-coated polystyrene particles as a solid phase. We have found that LPS-coated polystyrene particles, when used to prepare the standard curve, gave considerably less variability in the BCFIA than whole bacterial cells. These standards will remain stable for 6 months when stored at 4°C. In addition to standards older than 6 months, repeated warming and cooling of the standards may result in loss of reactivity of the antigen-coated beads in the immunoassay and could lead to erroneous results. Therefore, it is most important that identical aliquots of standards be prepared and then that one of the aliquots be warmed to room temperature when it is used in the immunoassay. When it is necessary to prepare a new preparation of antigen-coated beads, then it would be necessary to calibrate these beads against a known number of the homologous specific bacterial cells. Also, bacteria in plaque samples have a tendency to aggregate, which may reduce the sensitivity of the BCFIA. Resuspending the plaque sample in the presence of a mild detergent, 0.05% Tween, was critical in reducing aggregation of the bacteria. Moreover, prior to the immunoassay, washing plaque samples twice in 0.05% Tween removed extraneous debris inherent to plaque and also significantly reduced variability in the BCFIA.

To develop estimates of variability in the precision of the BCFIA, selected pooled plaque samples were repeatedly assayed. One acceptable method used to examine precision in an immunoassay is to determine percent coefficients of variation for repeated runs of the assay (7). That we can consistently obtain coefficients of variation of <10% in the inter- and intraassay experiments indicates that this immunoassay has good precision and would be useful in reliably detecting any of the five pathogenic bacteria studied in plaque samples. In demonstrating the utility of using bacteria as solid-phase matrices for antibody characterization in the BCFIA, other investigators also reported excellent reproducibility, with coefficients of variation similar to those reported here, e.g., <10% (19).

Numerous studies have implicated *P. gingivalis*, *B. intermedius*, *A. actinomycetemcomitans*, *E. corrodens*, and *F. nucleatum* in human periodontitis. Other investigators have already used species-specific MAbs to detect these periodontal pathogens in plaque, and some have suggested the potential clinical significance of using MAbs as an adjunct in the diagnosis of individuals at risk of developing periodontal disease (1, 2, 10, 11, 16, 20). The BCFIA described here is a highly sensitive and relatively quick assay for the quantitation of specific bacteria in plaque specimens. As many as 400 plaque samples or other microbial preparations per day could be evaluated with the BCFIA for *P. gingivalis*, *B. intermedius*, *A. actinomycetemcomitans*, *E. corrodens*, and *F. nucleatum*. Cultivable flora procedures, on the other hand, would take weeks or months to quantitate plaque samples for these bacteria similarly. This immunoassay could have important implications from both a research and a clinical perspective with respect to evaluating pathogenic microorganisms in plaque. The BCFIA should facilitate much needed longitudinal investigations of bacterial risk factors in human periodontal disease initiation and progression. Furthermore, the BCFIA would also seem to have a potential wide application to the analysis of complex microbial communities in general.

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